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13. ABSTRACT (Maximum 200 Words)

This project focuses on the potential role of the calcium-selective ion channel, CaT1, in prostate cancer progression. Although the physiological role of CaT1 is still poorly understood, it may be a "store-operated" calcium pore, a type of channel that is known to play a role in cell growth and survival regulation. The Aims of this project are to (1) Develop specific antibodies against CaT1 and characterize its expression pattern(s) in human prostate cancer tissues; and to (2) Determine the role of CaT1 in cell growth and survival regulation in human prostate cancer cells. We have successfully completed most of the objectives in Aim (Task) 1. During the first funding year, we developed and characterized a CaT1 antibody suitable for highly specific detection of the CaT1 protein in tissues. We used this novel reagent to characterize the expression patterns of CaT1 protein in mouse and human; we discovered that CaT1 is expressed by a number of exocrine organs; and we discovered that CaT1 is overexpressed in prostate, breast, thyroid, colon and ovarian carcinomas. These findings indicate that CaT1 is likely to serve as a component of transcellular calcium transport mechanisms in many tissues and epithelial cancers.

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INTRODUCTION

This project focuses on a recently discovered protein, CaT1, originally found in intestinal cells but which is now known to be present in a number of tissues. Gene expression analysis indicates that CaT1 is present at high levels in the prostate (10-50-fold higher levels than in most other organs). The physiological role of CaT1 is unknown; however, it has been functionally characterized by cloning, gene transfer and electrophysiological measurements in cell lines and frog eggs. These experiments have revealed that CaT1 is a calcium-selective channel of the outer cell membrane. It appears to function as a calcium entry pore, whereby ionic calcium enters the cell in response to defined triggers. CaT1 is a not the type of calcium channel that is "voltage-gated", such as those present in electrically excitable cells. Evidence indicates, however, that CaT1 may be a type of "store-operated " calcium pore. These types of channels are known to play a role in cell growth and survival regulation.

Our laboratory recently demonstrated that expression of the CaT1 gene is associated with human prostate cancer progression (Peng et al., 2001). Experiments with prostate cancer cell lines also indicate that CaT1 expression increases under conditions of androgen deprivation. These findings, along with the known properties of CaT1 and store-operated calcium channels, indicate that CaT1 may play an important functional role in regulating prostate cancer cell survival, including under conditions of androgen suppression. The link between a calcium entry channel and androgen-independent prostate cancer is highly novel, and potentially related to mechanisms of progression of hormone-insensitive cancer and to epidemiological data pointing to increased risk of prostate cancer from consumption of dairy products.

In this Idea Development Award, we proposed two primary objectives: (1) develop new probes (antibodies) to study the CaT1 protein in tissues; and (2) use molecular and cell biological methods to explore the functional role of CaT1 in prostate cancer cells.

BODY

Task 1. Develop specific antibodies against CaT1 and characterize its expression pattern(s) in human prostate cancer tissues.

Task 1 has largely been accomplished. Details of the development and use of CaT1 antibodies to study CaT1 expression in prostate and other tissues in human and mouse were reported to the DoD in our year 1 progress report. These studies resulted in the publication of an original research article in a major journal (Zhuang et al., 2002).

Task 2. Use molecular and cell biological methods to explore the functional role of CaT1 in prostate cancer cells.

Our published studies have linked CaT1 to aggressive (hormone-refractory) prostate cancer. Other recent studies from our group have demonstrated that prostate cancer cells employ a cholesterol-rich plasma membrane (lipid raft) compartment to transmit cell growth and survival signals (Zhuang et al. 2002; Kim et al 2004). Consequently, we have carried out a series of experiments to assess whether signal transduction mechanisms employing CaT1 use lipid raft microdomains as part of the signaling process. In the past year we have accumulated substantial unpublished data

to indicate that this hypothesis may be true and that CaT1 may play a major role in proliferative and survival signals relevant to prostate cancer progression. Attempting to identify such a mechanism was a major goal of the original grant proposal.

Finding 1: CaT1 localizes to prostate cancer cell lipid rafts

Lipid rafts are cholesterol- and sphingolipid-enriched microdomains in cell membranes, which have been proposed by us and others to serve as platforms for various cell signaling and transport processes. To examine the subcellular localization of CaT1 and explore its potential functional relationship with lipid raft microdomains in prostate cancer cells, we isolated lipid raft membranes from LNCaP prostate cancer cells by flotation in sucrose gradients and probed gradient fractions with antibodies for known raft proteins, such as Fyn and Giα2 (Figure 1). (Identification of CaT1 protein in these experiments was possible because we developed a highly specific anti-CaT1 antibody from the studies funded by this grant). We found that most of the CaT1 protein floated in sucrose gradients and co-localized with known raft marker proteins, indicating that CaT1 is present predominantly in lipid rafts in LNCaP cells. The observation that CaT1 localizes to lipid raft membranes implies a functional dependency between this cholesterol-rich membrane compartment and CaT1-mediated calcium influx.

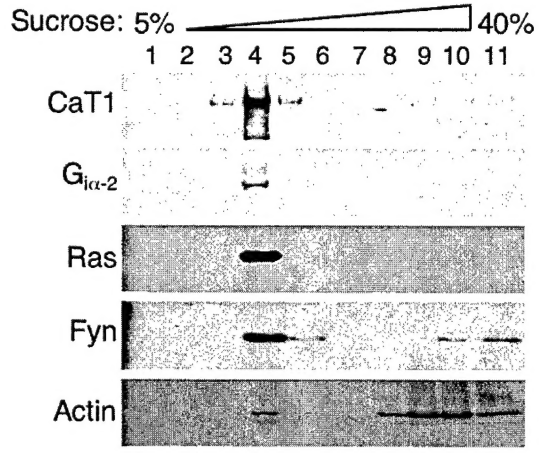

Figure 1. ***CaT1 localizes to light buoyant density lipid raft fractions in sucrose gradients.*** LNCaP cells were lysed using a detergent-free method and prepared for sucrose density ultracentrifugation. CaT1 localizes in the gradients with known lipid raft markers, including Giα2 and the Src-like kinase, Fyn.

Finding 2: Regulated calcium influx is potentiated by cholesterol addition to cell membranes

To further investigate the novel possibility that calcium entry is regulated by a lipid raft-dependent mechanism, we manipulated a key raft component, cholesterol, in calcium influx experiments. In experiments where store-operated calcium influx was triggered by thapsigargin (an inhibitor of the endoplasmic reticulum calcium pump), addition of exogenous cholesterol to cell membranes dramatically potentiated calcium influx (Figure 2). This effect was inhibited by the cholesterol-binding reagents, methyl-β-cyclodextrin and filipin. Further, the inhibitory effect of the calcium-binding compounds was completely reversed by restoration of membrane cholesterol levels by addition of exogenous cholesterol. These experiments are consistent with the data shown in Figure 1, where CaT1 was found to localize to lipid raft microdomains, and they suggest that regulated influx of calcium ions in LNCaP cells is mediated by cholesterol-rich lipid raft microdomains.

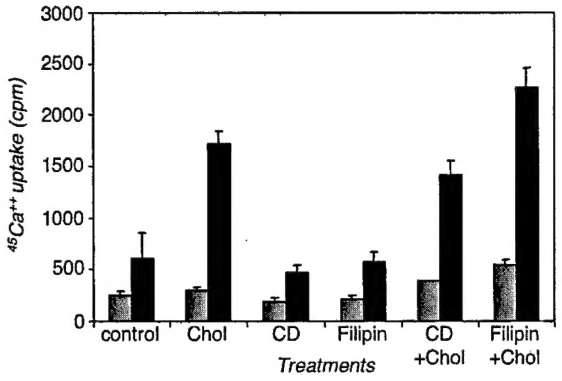

Figure 2. ***Cholesterol potentiaties store-operated calcium influx in LNCaP cells.*** Experimental groups were as follows: (1) control: (+/- thapsigargin treatment; (black bars are thapsigargin-treated in all conditions); (2) cholesterol pre-treament for 1 h (Chol); (3) 20 mM cyclodextrin treated for 1 h after cholesterol pretreatment (CD); (4) 2μg/ml filipin treatment for 1 h after cholesterol pre-treatment; (5) cholesterol reloaded after treatment with CD; (6) cholesterol reloaded after treatment with filipin. After the above manipulations, cells were harvested and $3x10^5$ cells were used for thapsigargin-induced calcium uptake assay according to Peng et al. J. Biol. Chem. 274:22739-22746, 1999.

Finding 3: Regulated calcium influx is dependent on intact lipid rafts

Calcitonin is a calciferic hormone that is secreted by parafollicular cells of the thyroid gland. Its function is to maintain calcium homeostasis by several mechanisms, including inhibiting osteoclast activity in bone, decreasing absorption of calcium ions from the small intestine, and decreasing calcium excretion from the kidney. Its possible role as a trophic factor involved in prostate cancer growth is unknown. We have now demonstrated that calcitonin upregulates CaT1 levels in a dose-dependent manner in the LNCaP cell line (Figure 3), providing a link between this hormone and a calcium-dependent signaling mechanism in human prostate cancer cells. Interestingly, calcitonin also activated signaling from the epidermal growth factor receptor family members EGFR/ErbB1 and ErbB2. These data provide evidence for a link between calcium regulation and well-known cell proliferation and survival pathways in prostate and other cancer cells.

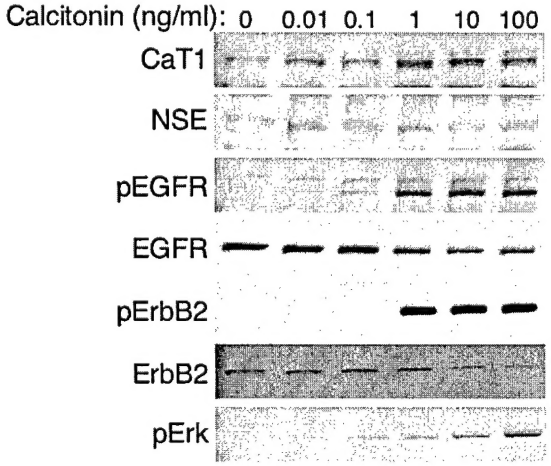

Figure 3. ***Calcitonin induces CaT1 expression and activates EGFR family receptor tyrosine kinases.*** pEGFR and pErbB2 indicate phosphorylated forms of the two receptors detected by phospho-site-specific western blot. pERK refers to the phosphorylated form of the ERK mitogen activated protein kinase. NSE=neuron-specific enolase.

REPORTABLE OUTCOMES

To date, four original articles have been published as a result of funding from this grant:

Zhuang, L., Peng, J-B., Tou, L., Takanaga, H., Adam, R.M., Hediger, M.A., and **Freeman, M.R.** (2002) Calcium-selective ion channel, CaT1, is apically localized in gastrointestinal tract epithelia and is aberrantly expressed in human malignancies. Laboratory Investigation 82:1755-1764.

Freeman, M.R., and Solomon, K.R. (2004) Cholesterol and prostate cancer. Journal of Cellular Biochemistry 91:54-69.

Cinar, B., Yeung, F., Konaka, H., Mayo, M.W., **Freeman, M.R.**, Zhau, H.E., and Chung, L.W.K. (2004) Identification of a negative regulatory cis-element in the enhancer core region of the Prostate Specific Antigen (PSA) promoter: Implications for intersection of androgen receptor and NF-kappaB signaling in prostate cancer cells. Biochemical Journal [Epub ahead of print—Jan 9, 2004].

Kim, J., Adam, R.M., Solomon, K.R., and **Freeman, M.R.** (2004) Involvement of cholesterol-rich lipid rafts in interleukin-6-induced neuroendocrine differentiation of LNCaP prostate cancer cells. Endocrinology 145:613-619.

KEY RESEARCH ACCOMPLISHMENTS AND CONCLUSIONS

We believe that we have now made the first link between regulated, store-operated calcium influx and cholesterol- and growth factor-dependent regulatory mechanisms in prostate cancer. These results relate directly to several hypotheses we sought to test in the original proposal, including the possible influence of diet (e.g., calcium, cholesterol) on regulatory mechanisms involved in prostate cancer progression. We anticipate that by the end of the grant period we will have accumulated sufficient data to compete successfully for an NIH R01 grant on calcium-dependent regulation of prostate cancer growth and survival mechanisms.

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Kim, J., Adam, R.M., Solomon, K.R., and **Freeman, M.R.** (2004) Involvement of cholesterol-rich lipid rafts in interleukin-6-induced neuroendocrine differentiation of LNCaP prostate cancer cells. Endocrinology 145:613-619.

Peng, JB, Zhuang, L, Berger, UV, Adam, RM, Williams, BJ, Brown, EM, Hediger, HA, and **Freeman, M.R.** (2001) CaT1 expression correlates with tumor grade in prostate cancer. Biochem. Biophys. Res. Comm. 282:729-734.

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Note: We are no longer using Human Subjects on this project.

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Calcium-Selective Ion Channel, CaT1, Is Apically Localized in Gastrointestinal Tract Epithelia and Is Aberrantly Expressed in Human Malignancies

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The Urologic Laboratory (LZ, RMA, MRF), Department of Urology, Children's Hospital Boston, and Department of Surgery, Harvard Medical School, Membrane Biology Program (J-BP, HT, MAH) and Renal Division (J-BP, HT, MAH), Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, and Harvard-Thorndike and Charles A. Dana Research Laboratories (LT), Division of Bone and Mineral Metabolism, Department of Medicine, Beth Israel Medical Center, Harvard Medical School, Boston, Massachusetts

SUMMARY: CaT1 is a highly selective calcium entry channel that has been proposed to be responsible for apical calcium entry in the vitamin D-regulated transcellular pathway of Ca^{2+} absorption; however, the lack of a CaT1 antibody suitable for immunohistochemistry has prevented the direct testing of this hypothesis by the localization of CaT1 protein in the gastrointestinal tract and other tissues. In this study, we developed two CaT1 antibodies and have used them to establish for the first time that CaT1 localizes to the apical membrane of intestinal absorptive cells, thereby providing the first direct evidence that this protein is in fact an apical entry channel in the gastrointestinal tract. In addition, we found that CaT1 protein is highly expressed in a number of exocrine organs including pancreas, prostate, and mammary gland, suggesting an, as yet, unrecognized role in secretory epithelia. Finally, we found CaT1 protein to be present at elevated levels in comparison with normal tissues in a series of prostate, breast, thyroid, colon, and ovarian carcinomas, consistent with previous reports of up-regulation of CaT1 mRNA in prostate cancer tissues. Our findings indicate that CaT1 is likely to serve as a component of transcellular calcium transport mechanisms in many tissues and epithelial cancers. (*Lab Invest 2002, 82:1755–1764*).

Calcium, a critical component of teeth and bones, is one of the most important minerals in vertebrate physiology. The ionic form of calcium (Ca^{2+}) is also an intracellular messenger that mediates aspects of muscle contraction, nerve transmission, enzyme and hormone secretion, and many other biological processes, such as cell cycle regulation and programmed cell death. The Ca^{2+} concentration in the circulation and extracellular fluid is maintained fairly constant through the action of calcitrophic hormones on three major organs: the intestine, the kidney, and the bone, where calcium ions are absorbed, reabsorbed, and deposited, respectively.

Intestinal Ca^{2+} absorption is an important determinant of calcium homeostasis, as all bodily calcium is ultimately obtained from the diet through intestinal absorption. Ca^{2+} enters the circulation through a paracellular route via tight junctions between the absorptive cells of the gut and a transcellular route across enterocyte apical and basolateral membranes. When dietary Ca^{2+} concentration in the lumenal side is higher than the blood side, Ca^{2+} enters primarily through the paracellular route. When dietary calcium is low, the transcellular route is unregulated and becomes dominant in Ca^{2+} absorption. The transcellular pathway involves a calcium entry channel in the apical membrane and a calcium pump (PMCA1) in the basolateral membrane. Several lines of evidence indicate that the recently identified Ca^{2+} selective channel, CaT1, is responsible for apical entry of Ca^{2+} into enterocytes (Barley et al, 2001; Peng et al, 1999, 2000a; Van Cromphaut et al, 2001); however, subcellular and tissue localization of the CaT1 protein has not been described.

CaT1 and CaT2 (also known as ECaC) are the two Ca^{2+}-selective channels in the transient receptor potential V (TRPV) channel family, which consists of six members. The other members of this family are nonselective cation channels, including the VR1 receptor, a capsaicin and heat-gated channel, and the osmoreceptor OTRPC4/VR-OAC/VRL-2/Trp12, which are activated by decreases in osmolarity (Liedtke et al, 2000;

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Montell et al, 2002; Strotmann et al, 2000). These channel proteins have six membrane-spanning helices and a pore region and are structurally related to the TRP channels. CaT1 and CaT2/ECaC were originally isolated from rat intestine and rabbit kidney and have been proposed to be responsible for the Ca^{2+} entry step of transcellular Ca^{2+} absorption/reabsorption in the intestine and the kidney, respectively (Hoenderop et al, 1999; Peng et al, 1999). The two channels exhibit constitutive activity when expressed in *Xenopus laevis* oocytes and show saturation kinetics with K_m values in the <1-mM range. CaT1 generates an inwardly rectifying current and the current-voltage relationship indicates that hyperpolarized potentials favor ion transit through the channel. CaT1 is activated by low intracellular Ca^{2+} and inactivated at high concentrations. Interestingly, when CaT1 expression levels are not high, CaT1 is also activated by passive and active intracellular Ca^{2+} store depletion. The overall biophysical properties of CaT1 closely resemble those of the Ca^{2+} release-activated I_{CRAC} channel (Yue et al, 2001). Therefore, in addition to CaT1's role as an apical Ca^{2+} entry channel in the transcellular Ca^{2+} transport pathway, CaT1 might also serve as an integral component of the capacitative Ca^{2+} entry pathway in other cells.

The CaT1 and CaT2/ECaC genes are located on adjacent positions on human chromosome 7 (Muller et al, 2000a; Peng et al, 2001a); however, their tissue distributions at the mRNA level in mouse and human are distinct. CaT2/ECaC expression is restricted primarily to the distal tubules of the kidney, whereas CaT1 mRNA is expressed in the intestine, placenta, and exocrine organs such pancreas, prostate, salivary gland, and testis (Hoenderop et al, 2000; Muller et al, 2000b; Peng et al, 2000b, 2001a). The expression pattern of CaT1 mRNA in many exocrine tissues suggests that it plays a fundamental role in secretion. However, in the absence of information on the subcellular location of the CaT1 protein, its potential role in exocrine cell function cannot be assessed.

Elevation of CaT1 mRNA expression in human prostate cancer tissues was recently reported by us and by another group (Peng et al, 2001b; Wissenbach et al, 2001). However, because of the lack of a CaT1 antibody, the question of whether the CaT1 protein was similarly elevated was not addressed in these studies. Furthermore, it has yet to be investigated whether elevated CaT1 expression in tumor tissue is a phenomenon unique to prostate cancer or whether this may be a common theme in tumors of epithelial origin.

In this study, we investigated the distribution of the CaT1 protein in the gastrointestinal tract in mouse and human by means of immunocytochemistry and Western blot analysis. We also investigated the distribution of CaT1 protein in exocrine organs and studied its expression in tumor tissues. Our results show that CaT1 is apically localized in most digestive and exocrine tissues. We also provide evidence that elevation of CaT1 expression may be a common event in cancers of epithelial origin.

Results

Antibodies Against Human CaT1

A 19-amino acid peptide corresponding to the intracellular carboxyl terminus of CaT1 was used to generate affinity-purified anti-CaT1 antibodies in rabbit and chicken. The region of CaT1 corresponding to this peptide is highly conserved between human and mouse (95% identity, 18/19) but poorly conserved between CaT1 and CaT2/ECaC. Human and mouse CaT2/ECaC demonstrate only 25% (5/19) and 30% (6/19) identity, respectively, with the CaT1 peptide (Fig. 1A). The R65291 rabbit anti-CaT1 antibody decorated the plasma membrane predominantly when FLAG-tagged CaT1 was expressed in *X. laevis* oocytes following injection of CaT1 cRNA, consistent with the conclusion that CaT1 is a membrane protein with six membrane-spanning helices (Fig. 1B). Both rabbit anti-CaT1 and M2 anti-FLAG antibodies produced the same labeling pattern (Fig. 1B), suggesting that they recognize the same protein. This was further confirmed by the finding that both antibodies (anti-CaT1 and anti-FLAG) detected an identical 86-kD band in Western blots of lysate from FLAG-CaT1 expressing oocytes (Fig. 1C). This band was not detected using lysate from human CaT2/ECaC cRNA-injected oocytes (data not shown).

Having confirmed that the rabbit antibody detects CaT1 expressed in *X. laevis* oocytes and that the protein localizes to the plasma membrane as anticipated from its primary structure and functional characteristics, we next tested our ability to detect CaT1 in mammalian cells. HEK293 cells were transiently transfected with expression plasmids encoding an EGFP-CaT1 protein or unmodified EGFP, and the cells were sorted by flow cytometry. In cells expressing the EGFP-CaT1 fusion protein, green fluorescence was present in the plasma membrane (Fig. 1D). In the control cells expressing EGFP only, green fluorescent labeling was homogeneous throughout the cytosol. Immunoblotting with the rabbit anti-CaT1 and the CH2747 chicken anti-CaT1 antibodies and the anti-FLAG M2 antibody detected a specific CaT1 band in all blots (Fig. 1E). However, the chicken IgY antibody detected CaT1 with substantially lower background than the rabbit antibody. In addition to detecting CaT1, the rabbit antibody labeled a 160-kD protein present in HEK293 cells transfected with pIRES empty vector (IRES) as well as pIRES-CaT1-transfected HEK293 cells (FCaT1) (Fig. 1E). The 160-kD protein detected by the rabbit antibody was immunoprecipitated and identified by mass spectrometry as early endosomal antigen 1 (EEA1) (Mu et al, 1995), even though EEA1 appears not to contain sequence motifs that resemble CaT1. The reason for this cross-reaction is unclear; however, the R65291 antibody does not recognize an EEA1-like band in *X. laevis* oocytes.

To determine whether the chicken anti-CaT1 antibody recognizes the mouse protein, we expressed the mouse CaT1 tail fused to GST in *Escherichia coli*. Figure 1F demonstrates that the chicken antibody

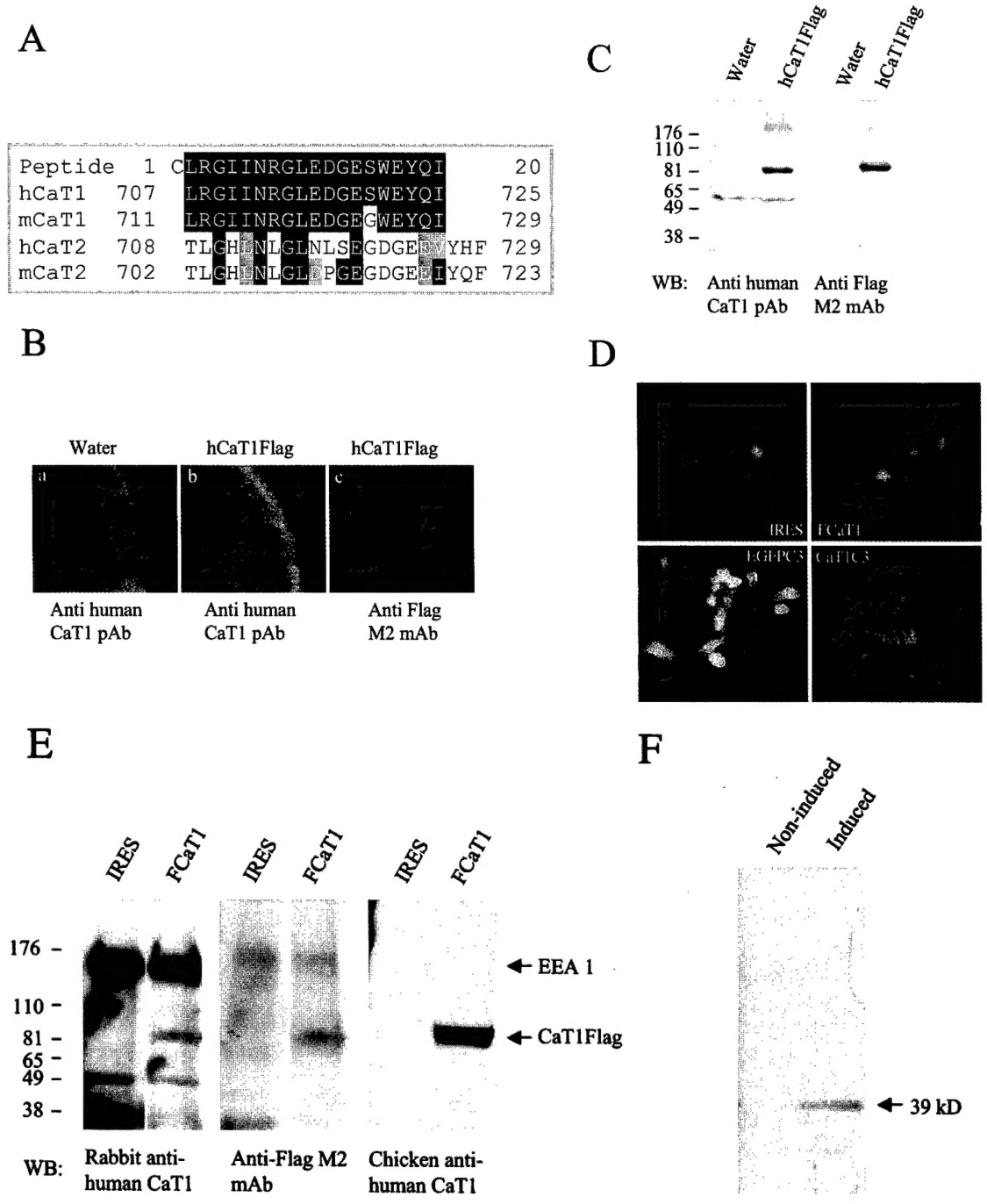

Figure 1.

Characterization of antibodies against CaT1. A, Alignment of the synthetic peptide and human CaT1, CaT2, mouse CaT1 and CaT2. B, Immunofluorescence labeling of human CaT1 expressed in *X. laevis* oocytes. (a) No labeling is visible on the membrane of a control oocyte labeled with the R65291 rabbit anti-serum. (b) Membrane labeling of FLAG-tagged human CaT1 expressed in *X. laevis* oocytes using R65291 rabbit anti-serum. (c) Labeling pattern, similar to that shown in (b), elicited by the anti-FLAG M2 monoclonal antibody in a section obtained from the same oocyte used in (b). In (a) and (b), FITC-conjugated IgG was used as secondary antibody, whereas in (c), Texas red-conjugated secondary antibody was employed. Original magnifications: ×200. C, A band of ~86 kD was detected with both polyclonal R65291 antiserum (1:500) and FLAG M2 monoclonal antibody (1:500) in samples of lysates from *X. laevis* oocytes expressing CaT1. The higher molecular weight bands in both blots likely represent dimers of human CaT1. No specific bands at the expected sizes were detected in samples from water-injected control oocytes. D, Membrane staining of human CaT1 in transfected HEK293 cells. When EGFP was expressed alone (ie, in the pIRES or pEGFP-C3 vector transfected cells, labeled as IRES and EGFPC3, respectively) or separately with CaT1 (ie, in pIRES-CaT1Flag transfected cells, labeled as FCaT1), EGFP was expressed in the cytosolic compartment, not in the membrane. In contrast, when EGFP was fused with CaT1, ie, in the pEGFP- C3CaT1 (CaT1C3)-transfected cells, green fluorescence was mostly detected on the plasma membrane. Original magnifications: ×200. E, Western blot analysis of CaT1 expression in HEK293 cells using R65291 and CH2747 antibodies. The lysates of HEK293 transfected with pIRES vehicle and pIRES-FCaT1 were fractionated by 4 to 20% linear gradient gel, electrotransferred to polyvinylidene difluoride membrane, then probed with R65291 (1:500), CH2747 (1:20,000), and FLAG M2 antibodies (1:500). The expected CaT1 bands were demonstrated in all three membranes in FCaT1 transfected cells. The prominent band at 160 kD observed in the rabbit R65291 anti-human CaT1 panel is due to cross-reaction with EEA 1 (see text for details). F, To determine whether the CH2747 chicken IgY reacts with mouse CaT1, a GST-mouse CaT1 C-terminus fusion protein was expressed in *E. coli*. The IPTG induced and noninduced control bacteria were lysed with Laemmli buffer and fractionated by SDS-PAGE. The electrotransferred polyvinylidene difluoride membrane was probed by CH2747 chicken IgY (1:20,000). The anticipated band of ~39 kD was observed only in IPTG-induced *E. coli*.

detected the fusion protein in IPTG-induced *E. coli*, whereas signal was not detected in samples from uninduced *E. coli*. The same band was also detected with an anti-GST monoclonal antibody (data not shown). This indicates that the chicken IgY recognizes both mouse and human CaT1 despite the fact that the serine at position 724 is occupied by glycine in the mouse CaT1 protein. The chicken anti-CaT1 antibody, which recognizes a single band on Western blots (Fig. 1E), was used for subsequent immunohistochemistry experiments.

Distribution of CaT1 in Mouse Digestive Tissues

A characteristic apical membrane staining pattern was revealed in the mouse digestive tract when this tissue was probed with the chicken anti-CaT1 antibody (Fig. 2). Positive labeling was detected in the esophagus, stomach, duodenum, ileum, cecum, and colon. CaT1 staining was essentially restricted to epithelial cells; no other tissue compartments demonstrated convincing staining above background. CaT1 staining localized predominantly to the apical epithelial membrane in all segments of the digestive tract except esophagus (Fig. 2, C–L, O, P). No basolateral labeling was detected in the mouse intestinal epithelia.

In the mouse pancreas, strong CaT1 labeling was detected in the exocrine acinar cells. No labeling was detected in islets of Langerhans. Within the pancreatic acinar cells, strong staining was restricted to the secretory pole, although much weaker basolateral staining was also observed (Fig. 2, M and N).

The specificity of the CaT1 immunofluorescent labeling was confirmed by Western blotting of mouse pancreas membrane extract. Chicken IgY against human CaT1 recognized mouse CaT1 in pancreas membrane extract but not in mouse liver membrane extract (not shown).

Distribution of CaT1 in Human Gastrointestinal Tract and Exocrine Glands

To confirm and extend our observation of CaT1 expression patterns in mouse gastrointestinal tract and

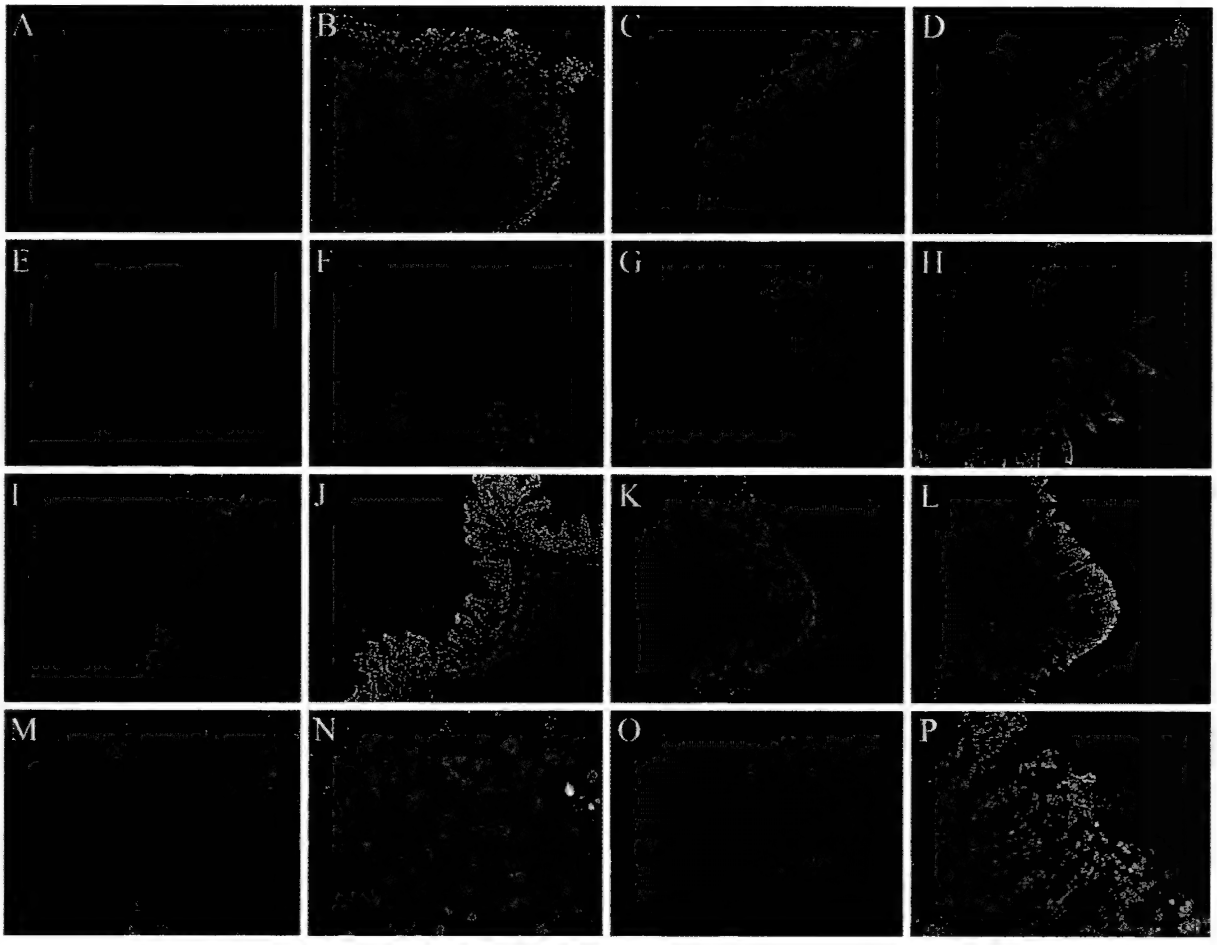

Figure 2.

Localization of CaT1 in mouse gastrointestinal tract and exocrine pancreas. Immunofluorescence labeling was employed on cryopreserved sections of mouse GI tract. The chicken IgY against human CaT1 was used at 1:100 dilution. The FITC-conjugated secondary antibody was used to visualize the expression pattern of the CaT1 protein. The labeling was demonstrated in the epithelial cells of esophagus (A), stomach (C), duodenum (E), ileum (G), cecum (I), and colon (K). The same view was also captured using another filter for DAPI staining; the DAPI-stained nuclei were blue and give the contour of the tissues: esophagus (B), stomach (D), duodenum (F), ileum (H), cecum (J), and colon (L). The labeling of pancreas is shown in M, where the apical surface of the acinar cells was stained. The merged images of CaT1 labeling and DAPI staining in pancreas, duodenum, and stomach are illustrated in N, O, and P. Original magnifications: A–L, ×100; M–P, ×400.

exocrine pancreas, we investigated the distribution of CaT1 in human gastrointestinal tract and in exocrine glands using commercially available slides. CaT1 expression was found in the epithelial cells of esophagus (Fig. 3, A and E), stomach (Fig. 3, B and F), small intestine (Fig. 3, C and G), and large intestine (Fig. 3, D and H), similar to the pattern observed in the mouse. In stomach, small intestine, and large intestine, CaT1 staining was largely confined to the apical epithelial cell surface. The stratified esophageal epithelia demonstrated strong CaT1 expression in the superficial layers (Fig. 3, A and E). One difference from the mouse was that membrane staining of CaT1 was observed in the deeper spherical epithelial cells in human esophageal sections (Fig. 3, A and E).

In the human exocrine pancreas (Fig. 3I), apical membrane and granular staining in acinar cells within the secretory pole was predominant. Very weak basolateral staining in acinar cells was also observed. Labeling became more obvious when the sections were stained for a longer period (data not shown). A serial pancreas section probed with preimmune chicken IgY as a negative control and in which no staining was detected is shown in Figure 3L.

Human exocrine glands in addition to the pancreas (breast tissue and sweat gland) were also stained with the chicken IgY. The mammary gland showed very strong staining on the inner surface of ductal epithelial cells and weak signals on the basolateral membrane (Fig. 3J). Sweat gland epithelial cells gave the same staining pattern; however, no difference in immunoreactivity was observed between the inner duct and basolateral surfaces (Fig. 3K).

Elevated Expression of CaT1 in Various Human Tumors

A commercial grid of human tumors and matched normal tissues was used to evaluate the expression of CaT1 in various cancer types. At least two cases of tumor and normal counterpart of each tumor type were examined. Images were captured in the light microscope under identical conditions. All normal tissues, including breast (A), ovary (B), thyroid (C), colon (D), and prostate (E), exhibited substantially weaker staining than their matched tumor tissues (Fig. 4, A1–E1). All of the tumor sections showed a histological pattern typical of invasive adenomas and a stronger immunohistochemical reaction than their normal tissue counterparts. We also observed that staining was weaker in one case of well-differentiated breast adenocarcinoma and thyroid adenocarcinoma in comparison with corresponding poorly differentiated tumors (data not shown). CaT1 was primarily localized to the cell membrane in breast and ovary tumors (Fig. 4, A2 and B2), while both cytoplasm and cell membranes stained positively in thyroid, colon, and prostate adenocarcinoma (Fig. 4, C2, D2, E2). Interstitial tissues adjacent to tumor cells were characteristically clear of CaT1 staining.

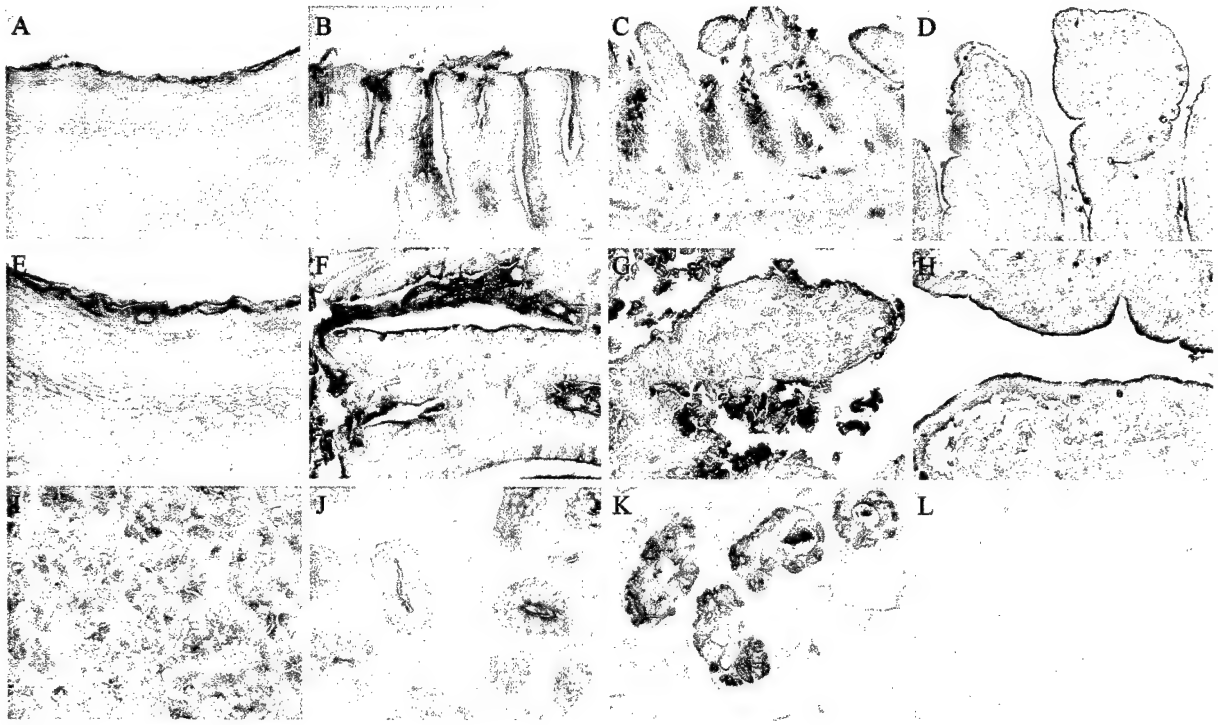

Figure 3.
Localization of CaT1 in human gastrointestinal tract and exocrine glands. The epithelia of esophagus (A, E), stomach (B, F), small intestine (C, G), large intestine (D, H), pancreas (I), and mammary gland (J) showed positive staining. The apical surface of the epithelia demonstrated the strongest staining intensity, and in some cases, apical cytoplasm also stained. Other tissue compartments (eg, muscle) demonstrated no staining. In sweat gland of the skin (K), both basolateral membranes and apical surface membranes demonstrated a strong positive reaction. A serial section of human pancreas showed no staining with preimmune chicken IgY (L). Original magnifications: A–D, ×200; E-L, ×400.

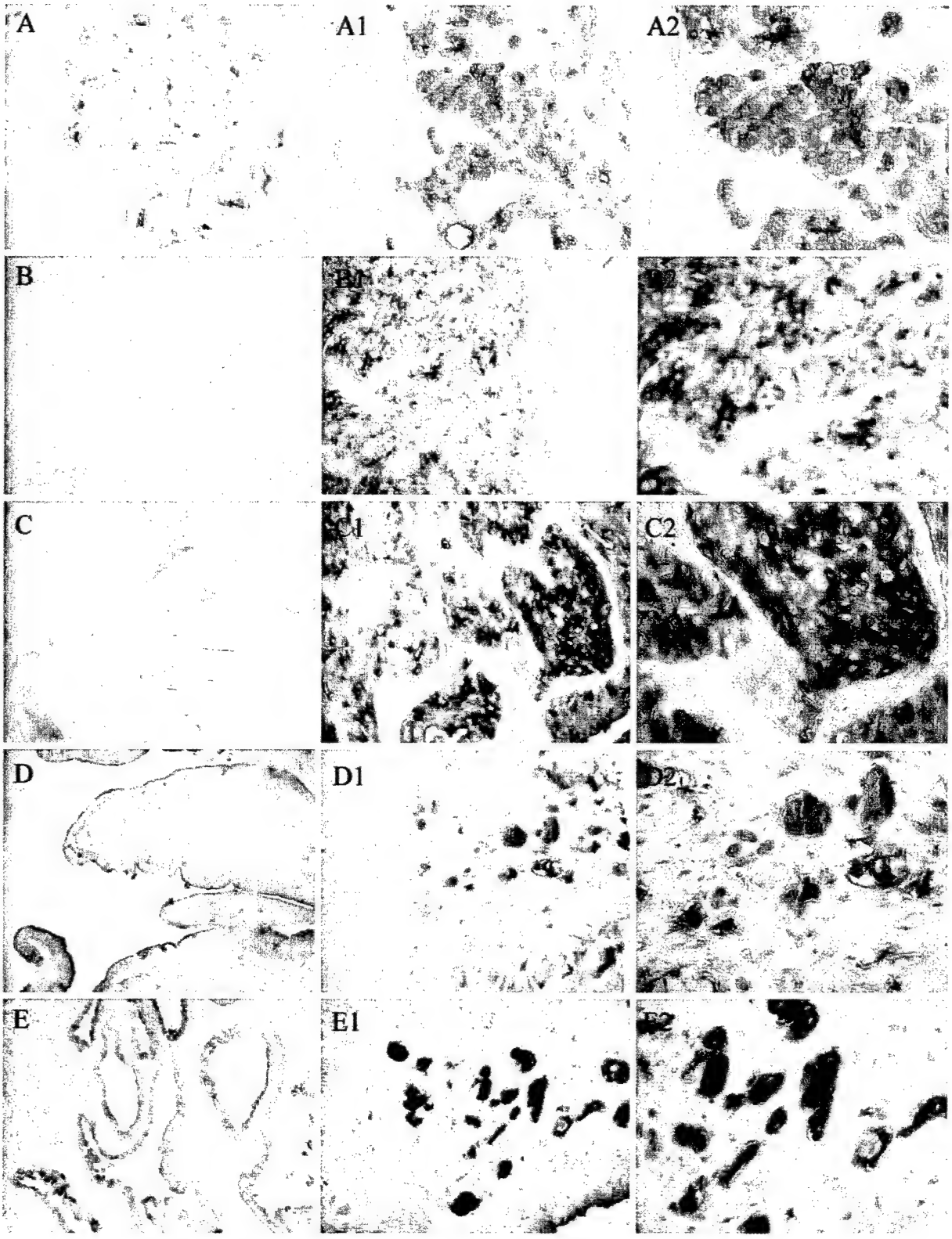

Figure 4.

Immunohistochemical staining of CaT1 in human normal and tumor tissues. The normal human mammary gland (A), prostate (E), and large intestine (D) showed positive staining by the epithelia. There was no specific staining in normal human ovary (B) and thyroid (C). The staining of mammary (A1), ovary (B1), thyroid (C1), colon (D1), and prostate (E1) adenocarcinoma demonstrated a stronger positive reaction than their normal counterparts. Magnified views of expression patterns of human CaT1 in A1–E1 tumor tissues are shown in A2-E2. Original magnifications: A–E, A1–E1, ×200; A2–E2, ×400.

Discussion

CaT1 has been proposed to be an apical entry channel in the transcellular pathway of calcium transport, which is the principal pathway of vitamin D-regulated calcium ion absorption (Barley et al, 2001; Peng et al, 1999; Slepchenko et al, 2001; Van Cromphaut et al, 2001; Wood et al, 2001). A recent study has also implicated CaT1 as a Ca^{2+} release-activated I_{CRAC} channel and, consequently, as potentially the primary mediator of store-operated calcium influx in a wide range of cells (Yue et al, 2001). Although CaT1 mRNA expression was evaluated previously in tissues and prostate tumors, no information about tissue or tumor distributions of the CaT1 protein has been published. In this study, we report the development of two CaT1 antibodies, including a monospecific anti-CaT1 chicken IgY antibody suitable for immunostaining of mouse and human tissues. We have used these new reagents to demonstrate for the first time (1) that CaT1 localizes to the plasma cell membrane in *X. laevis* oocytes, a cell type where it has been functionally characterized, and (2) that CaT1 is apically localized in cells of the gastrointestinal tract, thereby providing the first direct evidence for the hypothesis that CaT1 serves as an apical entry channel in digestive tract cells. We also (3) confirmed the expression of the CaT1 protein in several exocrine organs, and (4) we provide the first evidence that CaT1 upregulation appears to be a common feature in a variety of epithelial tumors in human.

The rabbit and chicken anti-CaT1 antibodies were generated against the last 19 amino acids of the human CaT1 carboxyl terminus. Both antibodies were able to discriminate between CaT1 and the closely related ion channel, CaT2/ECaC, in *X. laevis* oocytes. The CaT1 tail region was chosen for the immunogen peptide because this domain of the protein is not conserved in CaT2/ECaC. Although the immunogen peptide sequence differs by one amino acid residue between human and mouse, this difference did not diminish the chicken antibody's ability to recognize the mouse protein. Although generated via the same antigen, the chicken IgY was a significantly cleaner detection reagent than the rabbit antibody. Immunoprecipitation and mass spectrometric analysis demonstrated that the rabbit antibody cross-reacted with an abundant membrane protein, early endosomal antigen 1 (EEA1), and therefore this antibody was not used for immunostaining. The reason for this cross-reaction is unknown, as we could find little or no homology between the immunogen peptide and the EEA1 sequence. The greater evolutionary distance between chicken and human/mouse, in comparison with rabbit, is likely to account for the greater specificity seen with the chicken antibody.

The expression of CaT1 in gastrointestinal epithelial cells is consistent with the previously reported mRNA expression pattern (Peng et al, 2000b); however, our study is the first to report CaT1 expression throughout the entire digestive tract, from esophagus to colon. Localization of the CaT1 protein on the brush border apical surface of intestinal villi in duodenum, small intestine, and colon is consistent with CaT1's proposed role as a major transcellular mediator of Ca^{2+} transport out of the intestinal lumen. Other ion channels and transporters have been found to localize to the apical surface of enterocytes in a similar pattern to the one we have observed for CaT1 (Geibel et al, 2000; Marshall et al, 2002; Martel et al, 2000; Murer et al, 2001; Sangan et al, 2002).

Interestingly, stomach is generally considered not to play a major role in Ca^{2+} absorption; however, our study has shown strong CaT1 expression in mouse and human stomach. In the high-magnification view, stronger labeling was observed on the upper segments of the gastric glands in comparison with the lower segments (Fig. 2P). The epithelial cells lining most of the gastric surfaces and pits secret mucus to protect the epithelium from the acidic gastric fluid, while epithelial cells lining the lower segments of the gastric glands are primarily chief cells, which function to secret pepsin. We postulate that expression of CaT1 in mucus-secreting cells of the stomach may serve to restore intracellular Ca^{2+} balance after mucus secretion and consequent depletion of stored calcium pools (Yue et al, 2001).

Our study is also the first to report CaT1 expression in the mammary gland and in the sweat gland, consistent with its anticipated expression in pancreas, prostate, and salivary gland. These findings suggest a role for CaT1 in exocytotic secretory processes. Ca^{2+} influx constitutes a key stimulus for exocytosis in pancreas (Lang, 1999) and salivary gland (Ambudkar, 2000; Bird et al, 1998). Another potential function for CaT1 in exocrine epithelia would be in clearance/reuptake of the Ca^{2+} released into the acinar and ductal lumena following secretory events (Hug et al, 1996). CaT1 in the mammary gland and the prostate is primarily localized to the lumenal surface of the epithelial cells; however, in the sweat gland, CaT1 is located on both the lumenal and basolateral membrane surfaces (Fig. 3K). These differences may reflect a divergence in underlying physiological functions for Ca^{2+} influx in different exocrine organs. In murine and human pancreas, we also detected the expression of CaT1 on the basolateral surface of acinar cells in addition to high levels of expression in the apical membrane. Basolateral expression of CaT1 may be related to the need for calcium entry from the circulation.

We were surprised to find high levels of CaT1 expression in esophageal stratified epithelia. Because this part of the upper digestive tract is not known as a site of calcium absorption, this result is consistent with the view that CaT1 plays another functional role in vivo in addition to its calcium transport function. It is well known that calcium ions are important mediators of cell survival and cell death signaling programs, and stratified squamous epithelia are rapidly self-renewing cell populations. Consequently, CaT1 expression in stratified epithelia, as observed in mouse and human, suggests a potential role in cell proliferation and cell survival mechanisms that come into play following

tissue damage. CaT1 mRNA expression has been reported in murine skin (Weber et al, 2001). We also detected a CaT1 protein expression pattern in skin similar to the pattern we observed in the esophagus (data not shown), consistent with the hypothesis that CaT1 plays a role in rapid tissue renewal.

We also analyzed CaT1 expression in several human malignancies and their normal tissue counterparts. Although our sample size for each tumor type was small, an apparent increase in CaT1 protein was evident in breast, thyroid, colon, ovarian, and prostate carcinomas. This finding is consistent with two previous reports demonstrating that elevation of CaT1 mRNA expression correlates with features of aggressive disease in prostate cancer. In all tumors examined, CaT1 was confined to normal and cancer cells; specific labeling in the surrounding stroma was negligible. We believe that the apparent association between CaT1 expression and the cancer phenotype reported here should be studied in greater depth. Because Ca^{2+} flux is a prominent mediator of both survival and apoptotic signaling mechanisms in tumor cells, CaT1 overexpression could indicate a functional role for this protein in tumorigenesis, progression, or metastatic dissemination. Consistent with this assertion, we found that well-differentiated breast cancer and thyroid cancers expressed lower levels of CaT1 protein than their poorly differentiated counterparts. Our development of a CaT1 antibody suitable for semiquantitative immunostaining as well as Western blot analysis in whole-cell lysates allows these findings to be tested with more statistically robust sample populations.

In summary, our study for the first time has demonstrated expression patterns of the selective calcium entry channel, CaT1, in human and mouse tissues and in a series of human tumors. Our findings suggest that CaT1 serves as a major calcium transporter in the gastrointestinal tract and may be the primary mediator of calcium entry into the body. However, our results also suggest that CaT1 acts in other tissues, possibly in a homeostatic and protective role, and may also be a component of tumor cell proliferation and/or survival mechanisms. Further investigation into the physiological functions of CaT1 is ongoing. It is conceivable that molecular targeting of CaT1 may represent a novel therapeutic strategy for multiple malignancies via the regulation of calcium uptake.

Materials and Methods

Antibodies

Anti-serum R65291 was generated in rabbits against a peptide with a sequence corresponding to the last 19 amino acid residues of the carboxyl terminus of human CaT1. The peptide was synthesized by Biosource International, Inc. (Hopkinton, Massachusetts). Chicken IgY (anti-serum CH2747) was isolated and purified from the eggs of chickens that were injected and boosted with the identical synthetic peptide (Aves Labs, Tigard, Oregon). Other primary and secondary antibodies used are commercially available. These include anti-*Aequorea victoria* green fluorescent protein (GFP) polyclonal antibody (Living Colors A.v. peptide antibody) (Clontech, Palo Alto, California), anti-FLAG M2 mouse monoclonal antibody (Sigma, St. Louis, Missouri), fluorescein (FITC)-conjugated AffiniPure donkey anti-rabbit IgG and FITC-conjugated donkey anti-chicken IgY (Jackson ImmunoResearch, West Grove, Pennsylvania), and peroxidase-conjugated rabbit anti-chicken IgG and donkey anti-rabbit Ig G (Pierce, Rockford, Illinois).

Constructs

To express FLAG- and EGFP-tagged CaT1 in mammalian cells, human CaT1 cDNA with a FLAG tag positioned at the CaT1 N-terminus was introduced into pIRES and pEGFP-C3 vectors (Clontech) using a PCR approach. To accomplish this, two primers (5'-CAT TCT CGA GCA CCC CAT GGA CTA CAA GGA-3' and 5'-CAT TAG TCG ACT CAG ATC TGA TAT TCC CAG-3') were used to amplify the coding region of FLAG-CaT1. This DNA fragment was then introduced into the above vectors using the restriction enzyme sites (*Xho*I and *Sal*I) designed into the primers. The integrity of the constructs was confirmed by DNA sequencing. When transfected into mammalian cells, the two constructs produce FLAG-tagged human CaT1 (designated as FCaT1) and FLAG- and EGFP-tagged human CaT1 (designated as CaT1C3), respectively.

Expression of GST-Mouse CaT1 C-Terminus Fusion Protein in E. coli

The GST/mouse CaT1 C-terminus (636–729) fusion protein was cloned into pGEX4T-1 using *Eco*R I and *Xho*I sites. Briefly, the cDNA of the C-terminal region of mouse CaT1 was amplified by RT-PCR from Balb/c mouse cecum total RNA. The 5' primer was CAA GCC GAA TTC GAC AGG CAA GAT CTC AAC AGA CAA CGC A and the 3' primer was CAA CCG CTC GAG GTG AGA GCC AAC ATT CAG ATC TGG TAC TCC. The amplified cDNA was inserted into pCR-bluntII-TOPO by using bacterial strains (TOP10) (Invitrogen, Carlsbad, California). After digestion with *Eco*R I and *Xho*I (NEB, Beverly, Massachusetts), the insert cDNA was religated with pGEX4T-1 (Castle Hill, NSW, Australia) in bacterial strain DH5α. To express the fusion protein, the bacteria transformed with the recombinant protein were cultured with IPTG for 2 hours. After centrifugation, the pelleted bacteria were analyzed with SDS-PAGE (12.5% SDS-PAGE), and Western blotting was carried out with the CH2747 chicken IgY.

Cell Growth, Transfection, and Fixation

HEK293 cells were grown in Dulbecco's MEM (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37° C and 5% CO_2. The plasmids were prepared using the Maxi Plasmid Kit (Qiagen, Valencia, California). LipofectAMINE 2000 (Invitrogen) was employed as a DNA carrier for transient transfection

according to the manufacturer's specifications. Briefly, the DNA-liposome complex was prepared by mixing plasmids and LipofectAMINE 2000 in Opti-MEM I Reduced Serum Medium (Invitrogen) and incubating the mixture at room temperature for 20 minutes. The DNA-liposome mixture was then diluted with Opti-MEM I Reduced Serum Medium and added to 95% confluent HEK293 cells plated on six-well plates. After 5 hours of incubation at 37° C, the mixture was replaced with fresh DMEM supplemented with 10% serum. After 24 hours of incubation at 37° C and 5% CO_2, the cells were trypsinized and resuspended in 1 ml DMEM growth medium. Cells with high levels of green fluorescence were isolated by flow cytometry. The sorted cells were plated on six-well plates in the growth medium containing 100 U/ml penicillin and 100 U/ml streptomycin. Twenty-four hours later, medium was replaced with fresh DMEM containing 750 μg/ml G418 (Invitrogen). As cells reached 70–90% confluence, they were passaged into eight-well chamber slides (Falcon; Becton Dickinson, Franklin Lakes, New Jersey) and grown to 60–70% confluence by overnight incubation at 37° C, 5% CO_2. Followed by a brief wash in PBS, the slides were immersed into prechilled (−20° C) methanol for 5 minutes and then were mounted with Vectashield (Vector Laboratories, Burlingame, California) after a brief wash in PBS.

Expression of CaT1 in Xenopus laevis *Oocytes*

In vitro synthesis of capped CaT1 RNA, injection of RNA into *X. laevis* oocytes, and oocyte culture were performed as previously described[2,10]. Oocytes were embedded into optimal cutting temperature compound (Tissue-Tek, Tokyo, Japan) and kept at −80° C until used.

Whole-Cell Lysate Preparation

The transfected HEK293 cells and the *X. laevis* oocytes were rinsed twice with PBS and solubilized with 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, and COMPLETE protease inhibitor cocktail tablet (Roche, Mannheim, Germany). After 30 minutes incubation on ice, the insoluble material was removed by centrifuging the cell lysates at 15,000 rpm for 15 minutes at 4° C. Supernatants were collected as whole-cell lysates. The protein concentration was determined with the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, California).

PAGE and Immunoblotting

Equal amounts of total cell lysate from transfected HEK293 cells and *X. laevis* oocytes were solubilized in Laemmli sample buffer and subjected to SDS-containing polyacrylamide gel electrophoresis (PAGE) using a linear gradient precast gel (BMA, Rockland, Maine). The protein in the gel was subsequently electrotransferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Massachusetts). After blocking with 5% milk, the various anti-sera were used at the dilutions indicated in the figure legends. Immunocomplexes were visualized on film using horseradish peroxidase-conjugated secondary antibodies and the Supersignal Chemiluminescence System (Pierce, Rockford, Illinois).

Normal and Tumor Human Tissue Slides and Immunohistochemistry

The normal human digestive tissue set was purchased from Novagen (Madison, Wisconsin). Human NormalGrid and TumorGrid multitissue control slides were obtained from Biomeda (Foster City, California). On the slides were included human esophagus (n = 1), stomach (n = 3), small intestine (n = 3), large intestine (n = 3), pancreas (n = 6), breast (n = 2), thyroid (n = 5), skin (n = 3), ovary (n = 4), and prostate (n = 5). Adenocarcinoma tissues were from colon (n = 2), breast (n = 2), thyroid (n = 2), prostate (n = 3), and ovary (n = 2). The slides were deparaffinized in xylene and then rehydrated in gradient alcohol and water. After blocking in 5% BSA in PBS at room temperature for 30 minutes, the slides were incubated with anti-CaT1 chicken IgY CH2747 (1:300 diluted) overnight at 4° C. Following a 5-minute wash in PBS at room temperature (three times), a horseradish peroxidase-conjugated secondary donkey anti-chicken antibody was added at 1:1000 dilution and incubated at room temperature for 1 hour. Immunoreactivity was demonstrated using the DAB (3,3'-diaminobenzidine tetrahydrochloride) chromagen system (ABC Elite kit; Vector Laboratories). Controls for nonspecific immunoreactivity were performed with preimmune chicken IgY in place of anti-human CaT1 antibody.

Immunofluorescence Labeling of Mouse Tissues

Mouse (Balb/c) tissues were dissected and immediately washed three to five times in cold PBS, then embedded into optimal cutting temperature compound (Tissue-Tek, Tokyo, Japan). After sectioning on a cryostat at 5 μm, the slices were mounted on Super/Plus slides (Fisher Scientific, Pittsburgh, Pennsylvania), and slides were kept at 80° C until used. For immunostaining, slides were equilibrated at room temperature until no moisture was visible, then immersed in prechilled methanol (−20° C) for 5 minutes. After 5-minute washes (three times) in PBS, the slides were blocked with 10% milk in PBS for 30 minutes at room temperature. The CH2747 antiserum was used at 1:100 dilution in 2% BSA at room temperature for 1 hour. After washing, the secondary fluorescein isothiocyanate (FITC)-labeled anti-chicken IgY was added for 45 minutes. Slides were mounted with Vectashield with DAPI (Vector Laboratories) and were visualized under a fluorescence microscope (Olympus, Tokyo, Japan) equipped with a digital camera and controlled by a computer. Mice were maintained in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, and all experiments were conducted in accordance with the

principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

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Cholesterol and Prostate Cancer

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Abstract Cholesterol is a neutral lipid that accumulates in liquid-ordered, detergent-resistant membrane domains called lipid rafts. Lipid rafts serve as membrane platforms for signal transduction mechanisms that mediate cell growth, survival, and a variety of other processes relevant to cancer. A number of studies, going back many years, demonstrate that cholesterol accumulates in solid tumors and that cholesterol homeostasis breaks down in the prostate with aging and with the transition to the malignant state. This review summarizes the established links between cholesterol and prostate cancer (PCa), with a focus on how accumulation of cholesterol within the lipid raft component of the plasma membrane may stimulate signaling pathways that promote progression to hormone refractory disease. We propose that increases in cholesterol in prostate tumor cell membranes, resulting from increases in circulating levels or from dysregulation of endogenous synthesis, results in the coalescence of raft domains. This would have the effect of sequestering positive regulators of oncogenic signaling within rafts, while maintaining negative regulators in the liquid-disordered membrane fraction. This approach toward examining the function of lipid rafts in prostate cancer cells may provide insight into the role of circulating cholesterol in malignant growth and on the potential relationship between diet and aggressive disease. Large-scale characterization of proteins that localize to cholesterol-rich domains may help unveil signaling networks and pathways that will lead to identification of new biomarkers for disease progression and potentially to novel targets for therapeutic intervention. J. Cell. Biochem. 91: 54–69, 2004.

Key words: caveolae; lipid raft; HMG CoA-reductase inhibitor; chemoprevention; signal transduction

Abbreviations used: BPH, benign prostatic hyperplasia; cdk2, cyclin dependent kinase 2; CI, confidence interval; DIGs, detergent-insoluble, glycolipid enriched complexes; DRMs, detergent resistant membranes; FDA, US Government Food and Drug Administration; GPI, glycophosphatidylinositol; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IL-6, interleukin-6; LDL, low density lipoprotein; NSE, neuron specific enolase; PCa, prostate cancer; PI3K, phosphoinositide-3-kinase; PrEC, normal, primary culture prostate epithelial cells; PTEN, phosphatase and tensin homolog on chromosome 10; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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It has been known for about a century that cholesterol and other fatty deposits accumulate in solid tumors [White, 1909]. Increases in cholesterol content of prostatic adenomas relative to normal tissue was reported by Swyer 60 years ago [Swyer, 1942]. Since then, many studies of human subjects and animal models have supported the existence of a relationship between cholesterol in prostate tissues or secretions and benign and malignant prostate growth. Despite this long history, there have been few recent studies on the role of cholesterol in prostate cancer (PCa). Consequently, the physiological consequences, if any, of the accumulation of fat, and specifically of cholesterol, in relation to prostate carcinogenesis or progression are still poorly understood. This review will summarize the basic research and clinical observations that may relate to a functional role for cholesterol in PCa. We also present a testable model that attempts to unify many of the published observations pertaining to cholesterol and PCa progression. We propose that

this model provides a new approach toward the identification of novel molecular targets for PCa therapy.

PROSTATE CANCER INCIDENCE AND CHOLESTEROL

A variety of studies, beginning in the early 1980s, have linked increased risk of aggressive PCa to the consumption of animal products and/or fatty food. This association is still tentative, however, and the specific dietary components that may underlie such risks remain unknown. Nevertheless, it has been suspected for many years that life-style factors play a significant role in the rates of appearance and in the aggressiveness of clinically relevant PCa. Immigrants to the US and other Western nations from Asian countries, where the incidence of clinical PCa is typically low, show a dramatic increase in PCa detected clinically [Cook et al., 1999]. This increase in cancer incidence has been related to time of arrival, with increased cancer risk associated with early arrival in comparison to individuals who migrated later in life [Shimizu et al., 1991]. Because autopsy studies have shown that the incidence of occult PCa is similar in Asian and Western societies [Yatani et al., 1988; Pienta, 1994], the studies on immigrants point to an important role for exogenous factors, most probably diet, in PCa progression. Consistent with this, PCa incidence rates have recently risen in Asian countries that have been undergoing Westernization [Weisburger, 1997; Yang et al., 1999a]. Michaud et al. [2001] have reviewed the literature on diet and PCa incidence and have reported that, while association of PCa risk with the consumption of meat products has been relatively consistent (15 out of 19 studies reporting an association), studies examining fat intake have been less consistent. In a recent review [Kolonel et al., 1999], it was concluded that, while dietary fat may be related to PCa risk, "the specific fat components that are responsible are not yet clear."

As of this writing, the Michaud et al. prospective study is the most comprehensive analysis of the relationship between consumption of animal products and PCa risk. It involved 47,780 subjects in the Health Professionals Follow-up Study and demonstrated an elevated risk specifically of metastatic PCa and consumption of red meat and dairy products. In that analysis, there was no demonstrable association between animal products in the diet and total PCa, suggesting that the association is specifically with progression to metastatic disease. These investigators concluded that nutrients such as calcium and fatty acids explain much of the association between dairy products and metastatic PCa risk, but that the association with meat products cannot be explained by intakes of calcium, saturated fat, or α-linoleic fatty acids.

Cholesterol, a neutral lipid that plays an essential role in the maintenance of the integrity of biological membranes, is a prominent component of a diet containing animal products. In addition to its role in membrane structure, cholesterol also serves as a precursor in the synthesis of bile acids and many endocrine signaling mediators, such as the steroid hormones. Cholesterol is synthesized in mammalian cells via the mevalonate pathway (Fig. 1), which also produces a number of other important biochemical end-products. Isoprene units, produced by the mevalonate pathway, are precursors in the synthesis of a variety of molecules, including proteins, which are modified post-translationally. Isoprenoid modification of signaling proteins, such as Ras and Rho family members, are essential for proper membrane targeting of these molecules. Isoprenylated proteins participate in signal transduction pathways that regulate diverse processes such

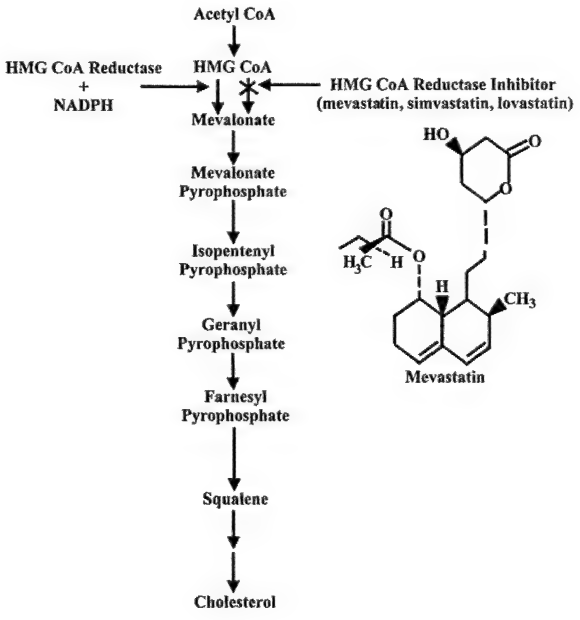

Fig. 1. The mevalonate pathway.

as the cell cycle, cell survival mechanisms and cell motility. Mevalonate products are thus essential for a wide-range of biological activities, from hormonal regulation of endocrine target organs to electron transport. The complexity and diversity of products originating from the mevalonate pathway have confounded studies focused on potential relationships between circulating cholesterol levels, cholesterol intake by diet or pharmacologic management of circulating cholesterol in cancer incidence or progression.

Most epidemiological studies have not found an association between circulating cholesterol levels, whether or not linked to diet, and cancer risk [Wu et al., 1994; Veierod et al., 1997; Chen et al., 2002; Smith-Warner et al., 2002]. This is consistent with the current state of the literature in which links to intake of fat and cancer incidence at most organ sites are modest. However, there are exceptions to this general rule. Several studies have reported statistically significant correlations between cholesterol intake and cancer risk [De Stefani et al., 1997; Horn-Ross et al., 1997; Jarvinen et al., 2001]. These findings are consistent with the possibility that prolonged consumption of cholesterol-rich foods might promote progression of certain cancer types or cancer growth in select tissues.

Some studies have reported an inverse association between cancer incidence and cholesterol levels for certain neoplasms [Kaplan et al., 1997]. Evidence suggests that this negative relationship is likely attributable in many cases to hypocholesteremic effects of pre-existing cancer [Knekt et al., 1988; Wald et al., 1989]. Although the question of the effect of undetected, pre-existing cancer on circulating cholesterol can be debated [Vatten and Foss, 1990], it is clear that frank cancer is indeed associated with lower circulating cholesterol levels in human patients [Umeki, 1993; Eichholzer et al., 2000; Fiorenza et al., 2000]. This negative association provoked long-term studies designed to identify potential health risks to patients on cholesterol-lowering therapy for cardiovascular disease. The results of several such studies indicate that chronically lowered cholesterol does not increase cancer risk [Waters, 2001; Heart protection study collaborative group, 2002] and may, in fact, lower cancer incidence at many organ sites [Blais et al., 2000; Pedersen et al., 2000].

HMG-CoA REDUCTASE INHIBITORS AND CANCER

The above discussion makes it clear that attempts to use epidemiological tools to assess any potential association between dietary or circulating cholesterol and risk of clinical PCa are confronted with significant challenges. Several older studies that attempted to establish a link between serum cholesterol levels and PCa risk did not report an association [Hiatt and Fireman, 1986; Knekt et al., 1988; Smith et al., 1992]. Another approach is to ask whether long-term treatment with cholesterol-lowering drugs affects PCa detection rates, incidence of aggressive disease, or disease-specific survival. These questions are only now beginning to be addressed.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, more commonly known as "statins," are cholesterol-lowering drugs that have been widely used for many years to reduce the incidence of adverse cardiovascular events. HMG-CoA reductase catalyzes the rate-limiting step in the mevalonate pathway (Fig. 1) and these agents lower cholesterol by inhibiting its synthesis in the liver and in peripheral tissues [Koga et al., 1990]. HMG-CoA reductase inhibitors function at an early step in the synthesis of cholesterol; as a consequence, the levels of cholesterol, and its upstream precursor isoprenoids, geranylgeranylpyrophosphate and farnesylpyrophosphate, are reduced. Thus, essential cell components that require isoprenoids, e.g., dolichols and ubiquinone (a polyisoprenylated quinoid cofactor of the electron transport chain), are affected by HMG-CoA reductase inhibitors. Statin drugs (e.g., pravastatin, lovastatin, simvastatin) now have a sufficiently long clinical history so that safety concerns for many of them can be definitively evaluated. Toxicity during long-term therapy with most statins is minor and recent studies have reported significant general health benefits with prolonged statin therapy [Pedersen et al., 2000; Waters, 2001].

A prospective analysis of the effect of long-term HMG-CoA-reductase inhibitor therapy on PCa incidence or progression rates using modern methods of study design has not yet appeared in the peer-reviewed literature. However, the results of a large-scale study evaluating the effect of long-term statin therapy specifically on cancer incidence rates was reported at the

annual meeting of the American Society for Clinical Oncology in 2003 [Graaf et al., 2003]. That study, the report of which has to date only appeared in abstract form, was conducted by researchers from the Academic Medical Center at the University of Amsterdam. The study examined 20,000 patients, comparing those taking statins with those taking other cardiovascular protective drugs within the period 1983–1998. These investigators found a 20% reduction in total cancer incidence (adjusted odds ratio = 0.80; 95% CI = 0.66–0.96) in the statin cohort with the largest reductions in the incidence of prostate and kidney cancer. Graaf et al. found statins to be protective when used longer than 4 years (adjusted OR 0.64; 95% CI: 0.44–0.93) or when more than 1350 Defined Daily Doses were taken (adjusted OR 0.60; 95% CI: 0.40–0.91). Interestingly, patients that terminated statin therapy returned to a baseline level of risk within 6 months. At this writing, the evaluation of these data awaits peer review; however, if verified, this finding suggests the possibility that inhibiting HMG-CoA-reductase may have clinical benefit in the chemoprevention of PCa.

Pedersen et al. [2000] conducted a randomized, placebo-controlled study of cause-specific mortality rates in patients on long-term (up to 8 years) simvastatin therapy. These investigators reported fewer deaths from cancer in the simvastatin group in comparison to the placebo group, although the difference was not considered significant. In a recent nested case–control study addressing potential risks of HMG-CoA-reductase inhibitor therapy with respect to potential increases in cancer incidence, nearly all cancer sites examined were either not associated, or were inversely associated, with statin therapy [Blais et al., 2000]. Interestingly, in this study PCa incidence declined in the HMG-CoA-reductase inhibitor group (adjusted rate ratio = 0.74; CI = 0.36–1.51) in comparison to the referent group (patients taking bile acid-binding resins). Anti-cancer efficacy of statins in comparison to other methods of cholesterol lowering may arise from the fact that these agents not only lower serum cholesterol but, in addition, reduce cholesterol synthesis in peripheral tissues as well as in the liver. This may be of considerable benefit in the case of prostatic neoplasms because the prostate has been reported to synthesize cholesterol at a rate even higher than the liver [Schaffner, 1981].

HMG-CoA-reductase inhibitors have been demonstrated to exert potent anti-cancer effects in model systems. A recent review has summarized the relevant publications on this topic [Chan et al., 2003]. Studies with cell culture models indicate that statin drugs can inhibit cancer cell growth and motility [Jani et al., 1993; Farina et al., 2002], induce apoptosis [Wong et al., 2001; van de Donk et al., 2002] and inhibit endothelial cell migration and tube formation, properties associated with angiogenesis [Vincent et al., 2001; Park et al., 2002]. Mevastatin, for example, has been shown to inhibit cell cycle progression in PC-3 human PCa cells by inhibiting cyclin dependent kinase (cdk2) phosphorylation [Ukomadu and Dutta, 2003]. Animal studies have verified that this class of agents has a substantial capability to retard tumor growth [Narisawa et al., 1994; Alonso et al., 1998], in vivo angiogenesis [Park et al., 2002] and tumor metastasis [Jani et al., 1993; Alonso et al., 1998; Farina et al., 2002]. In general, the statins also exhibit a robust selectivity for tumor cells over normal cells [Wong et al., 2001], an essential attribute for successful cancer therapy. Their ability to enhance the efficacy of conventional chemotherapeutic agents has also been demonstrated [Lishner et al., 2001; Wachtershauser et al., 2001]. Because most of the statins are now known to be well-tolerated by patients, and because they affect many processes governing the behavior of malignant cells (via multiple downstream effects on the mevalonate pathway), continued evaluation of these compounds in clinical trials as potential chemopreventive agents or as adjuvants to standard therapy is warranted. However, general conclusions about the anti-cancer effectiveness of the statins is not advised because the different compounds can exhibit significantly different activity profiles against tumor cells [Wong et al., 2001]. This difference in potency between various statins may account for reports claiming no effect of statin use on cancer incidence [Coogan et al., 2002].

CHOLESTEROL CONTENT OF PROSTATE CANCER CELLS AND TUMORS

Cells in the prostate, as is the case with other tissues, synthesize cholesterol endogenously via the mevalonate pathway. However, much of the cholesterol residing in cell membranes originates from the uptake of circulating lipoproteins [Simons and Ikonen, 2000]. Consequently,

cellular cholesterol content is a balance between metabolic mechanisms intrinsic to the cell and the regulatory functions of cholesterol distribution in the organism. Cholesterol content of cell membranes is under tight homeostatic regulation and involves synthetic pathways in the endoplasmic reticulum, transfer of cholesterol from lipoproteins to the exoplasmic leaflet, receptor-mediated internalization, several intracellular transport mechanisms, and extensive efflux from the cell via secretion of lipoprotein complexes. Extensive evidence indicates that this complex homeostatic mechanism breaks down in cancer and also in the aging prostate.

Swyer, using a histologic test, was the first to report that the cholesterol content of BPH tissues was higher (approximately double) than that of normal prostatic tissues [Swyer, 1942]. He also noted a spatial relationship between presumptive cholesterol accumulation and cellular hyperplasia, a finding similar to that reported by White in the early 20th century in an analysis of non-prostatic tumors [White, 1909]. Subsequent studies of human and animal prostate tissues also reported increases in cholesterol content in the prostate and in prostatic secretions correlating with disease, age, or the presence of malignancy [Schaffner, 1981]. These older observations are in agreement with recent studies of the relative cholesterol content of human breast cancers as evaluated by Raman spectroscopy (M. Feld, A. Haka, personal communication). Cholesterol accumulation may be a more general property of cancer and has been reported in a variety of tumor types [Dessi et al., 1992, 1994; Rudling and Collins, 1996; Yoshioka et al., 2000; Kolanjiappan et al., 2003]. Cancer-associated increases in tissue cholesterol content have also been reported to affect normal tissues surrounding malignant tumors [Nygren et al., 1997]. Cholesterol increases in tumor tissues likely occur by multiple mechanisms, including increased absorption from the circulation [Graziani et al., 2002; Tatidis et al., 2002], loss of feedback regulation through down-regulation of low density lipoprotein (LDL) receptors [Caruso et al., 1999] and up-regulation of components of the mevalonate pathway, particularly HMG-CoA reductase [Caruso et al., 1999; Caruso et al., 2002]. Androgen also stimulates lipogenesis in human PCa cells directly by increasing transcription of the fatty acid synthase and HMG-CoA-reductase genes [Heemers et al., 2001]. Other components of the mevalonate pathway, such as farnesyl diphosphate synthase, are also regulated by androgen and may play a role in accumulation of cholesterol and other lipid products in the prostate [Jiang et al., 2001]. Because cholesterol uptake and synthesis are coupled to the cell cycle [Wadsack et al., 2003], the link between cholesterol, other lipogenic mechanisms and androgen action suggests the possibility that lipid products of these pathways are involved in androgenic stimulation of PCa cell growth.

The first evidence that lowering cholesterol levels systemically might have the capability to alter prostate cell growth and/or survival was first presented by Schaffner and colleagues in a series of innovative studies. These investigators demonstrated that prostate regression could be selectively induced in dogs and rodents by oral application of hypocholesteremic agents, such as the polyene macrolide candicidin [Gordon and Schaffner, 1968; Schaffner and Gordon, 1968; Fisher et al., 1975; Schaffner, 1981]. Candicidin and structurally similar agents, such as amphoteracin B, exert biological effects by binding to cholesterol and closely related sterols [Charbonneau et al., 2001]. Oral administration of candicidin and similar agents likely lowers circulating cholesterol by inhibiting its absorption from the gut [Schaffner and Gordon, 1968]. Several human trials of oral candicidin for BPH in the 1970s reported symptomatic improvement [Keshin, 1973; Orkin, 1974; Sporer et al., 1975], with no alteration in hormonal status [Orkin, 1974], indicating that changes in the prostate, which included regressive histomorphologic changes within the gland [Keshin, 1973], were likely not the result of suppression of androgen production or utilization. Collectively, these studies suggest the intriguing possibility of manipulating prostate cell growth or homeostasis in situ by lowering circulating cholesterol levels pharmacologically.

CHOLESTEROL AND LIPID RAFTS

In the plasma membrane and other intracellular membranes, cholesterol accumulates in specialized structures known by various names, such as lipid rafts, detergent-resistant membrane domains (DRMs), and detergent-insoluble, glycolipid-enriched complexes (DIGs). Evidence for the existence of such cholesterol-rich membrane domains was first developed

from studies of glycosylphosphatidylinositol (GPI)-anchored proteins on lymphocytes and brush border membranes of the kidney and gut [Gunter et al., 1984; Hooper and Turner, 1987, 1988a,b]. These early studies described GPI-anchored proteins as being lipid-anchored and 'detergent-insoluble', yet capable of delivering cell signals when cross-linked. These findings were, however, paradoxical: how could proteins incapable of spanning the lipid bilayer transduce signals? This dilemma began to clarify when it was subsequently demonstrated that GPI-anchored proteins co-immunoprecipitated with both Src family tyrosine kinases [Stefanova et al., 1991; Shenoy-Scaria et al., 1992; Thomas and Samelson, 1992] and heterotrimeric G proteins [Solomon et al., 1996]. However, these observations led to a second paradox: how do proteins that do not span the bilayer interact with inner-leaflet signaling molecules? In the last decade this latter paradox has been largely resolved with the recognition that detergent-insoluble membrane domains serve as important nodes for signal transduction and other essential processes, such as cholesterol transport.

Studies into the composition of biological membranes that are resistant to solubilization in cold non-ionic detergents, such as Triton X-100 and Nonidet P-40, but which are not associated with the insoluble cytoskeleton, resulted in the discovery that discrete membrane subfractions contain high concentrations of cholesterol and fatty acids with long saturated acyl chains. Based on the apical vs. basolateral sorting properties of these domains, Simons and colleagues began to refer to these regions as 'patches,' and later as 'lipid rafts' [Simons and van Meer, 1988; Simons and Wandinger-Ness, 1990]. These and subsequent studies established that biological and artificial membranes that contain high concentrations of cholesterol and saturated fatty acid chains will spontaneously form "liquid ordered" aggregates, a heretofore theoretical condition that had been hypothesized to exist between the common liquid disordered state and the non-biological gel state [Pike, 2003]. In biological membranes, lipid rafts are enriched in sphingolipids (e.g., sphingomyelin and glycosphingolipids) relative to the majority of the membrane. Rafts are formed by self-aggregation of these lipids during their transport from the *trans*-Golgi network to the cell surface. These membrane patches are rich in proteins but likely represent only 10–15% of the plasma membrane area.

At least two morphologically distinguishable varieties of lipid raft exist on cell surfaces. The more familiar type has been named caveolae ("little caves") and are identifiable in electron micrographs as striated 50–100 nm invaginations in the plasma membrane [van Deurs et al., 2003]. Caveolae also exist as intracellular vesicles. Their invaginated and vesicular architecture is conferred by members of the caveolin protein family [Rothberg et al., 1992]. Caveolins, structural proteins that bind cholesterol, are necessary for caveolae formation but, because the biological function(s) of caveolae are still not well understood, their wider function is unclear. All three members of the mammalian caveolin family (caveolin-1, -2, and -3) have been knocked out in the mouse and, surprisingly, the functional deficits in these animals are relatively minor, given that loss of caveolin expression results in the complete ablation of an intracellular organelle [Galbiati et al., 2001a; Razani et al., 2001]. The second variety of raft has been named the flat raft or G domain. Flat rafts do not contain caveolin proteins and thus do not form a recognizable membrane structure identifiable by electron microscopy. Both type of lipid raft are isolated biochemically using similar approaches and have been shown to contain GPI-anchored proteins, Src family kinases, heterotrimeric G protein subunits, and other cell signaling molecules, such as receptor tyrosine kinases (RTKs) [Li et al., 1996; Solomon et al., 1996; Liu et al., 1997b; Rietveld et al., 1999]. Raft composition is likely to be dependent on cell type, although large-scale characterization of raft-resident proteins using proteomics approaches is just beginning [Bini et al., 2003; Foster et al., 2003]. Caveolae, which are present in adipocytes, myocytes, osteoblasts, endothelials as well as other cell types, are the most studied form of lipid raft to date.

Although the mechanisms of protein localization to rafts are still poorly understood, many involve post-translational modifications. Targeting mechanisms to rafts include the presence of a GPI-anchor, dual acylation (Src kinases and heterotrimeric G protein subunits) [Moffett et al., 2000] and linkage to cholesterol (Hedgehog) [Rietveld et al., 1999]. In contrast, prenylated proteins (e.g., Rap1, Rab5) may be excluded from rafts [Rietveld et al., 1999; Zacharias et al., 2002]. Other signaling mole-

cules identified in rafts in certain cell types include the endothelin receptor, thrombin receptors, multiple growth factor receptors, ion channels and pumps, an inositol 1,4,5-trisphosphate receptor, phosphoinositide-3-kinase (PI3K), and protein kinase C (PKC) isoforms [Chun et al., 1994; Schnitzer et al., 1995; Couet et al., 1997; Liu et al., 1997a,b; Bi et al., 2001].

Lipid rafts appear to serve a number of functions, such as intracellular transport and sorting of molecules, receptor down-regulation and recycling, and targeted export of proteins and lipids. In this review, which deals with the potential link between cholesterol and PCa, we will emphasize the role that lipid rafts are likely to play as essential platforms for signal transduction.

In cell signaling, rafts appear to act as a means of assembling components of specific pathways in ways that provide a regulatory architecture for transmission of signal. Rafts are believed to accomplish this by co-localizing cognate proteins so as to facilitate interactions and by excluding proteins capable of degrading signal, such as protein or lipid phosphatases. These functional properties of lipid rafts result in their ability to organize downstream signaling components in close proximity to surface receptors. They also create local environments in which signal propagation, amplification and cross-talk between pathways can occur. Some of the mechanisms by which lipid rafts may conceivably regulate signal transduction events are diagrammed in Figure 2 and include protein sequestration, assembly of pre-formed signaling complexes and intracellular and intramembrane trafficking and sorting. Proteins that have been implicated in signaling through lipid rafts include the T-cell receptor [Horejsi, 2003], the B-cell receptor [Saeki et al., 2003], integrins [Wary et al., 1998], ephrins [Bruckner et al., 1999], and the EGF receptor [Couet et al., 1997; Zhuang et al., 2002].

LIPID RAFTS AND PROSTATE CANCER

The first evidence linking lipid rafts to PCa was published by Thompson and colleagues, who identified caveolin-1 as a marker for aggressive PCa [Yang et al., 1998, 1999b; Tahir et al., 2001]. Subsequent studies from this group indicated that caveolin-1 is a predictor of poor outcome following surgery in lymph node-negative PCa patients [Satoh et al., 2003]. This literature has recently been reviewed [Mouraviev et al., 2002]. The relevance of these observations to our topic lies in the realization that caveolins localize essentially exclusively to lipid raft microdomains and are, in fact, the structural basis for the invaginated appearance of the caveolar form of raft. Consequently, a prominent marker of disease progression in PCa is also a marker for a cholesterol-rich membrane compartment. In addition, because caveolins may be involved in cholesterol transport to the cell membrane [Simons and Ikonen, 2000], higher caveolin levels may coincide with higher membrane cholesterol.

The link to caveolin-1 implicates the lipid raft microdomain as a potential site for signal transduction events relevant to PCa progression. The possibility that this association is functional, as opposed to simply correlative, is supported by the demonstration that anti-caveolin-1 antibodies suppressed PCa metastasis in mice, suggesting that caveolin-1 may play a direct role in metastatic dissemination [Tahir et al., 2001]. Caveolin-1 has also been shown to interact directly with the androgen receptor (AR) and appears to be capable of participating in the mediation of androgen-dependent signals in PCa cells [Lu et al., 2001]. Recent reports have demonstrated that members of the steroid hormone superfamily, including the androgen receptor [Sun et al., 2003], can function by a mechanism that is independent of their traditional role as transcriptional regulators (so-called "non-genomic" functions for these molecules), and that they can localize to rafts. These findings suggest the possibility that lipid rafts may regulate PCa cell growth and survival functions by compartmentalizing signaling proteins involved in hormonally responsive or dependent pathways, e.g., steroid hormone receptors. Although this possibility has only begun to be explored, recently published papers suggest this is going to be an extremely fruitful area of inquiry in studies of signal transduction by steroid hormones [Boonyaratanakornkit et al., 2001; Lu et al., 2001; Chambliss et al., 2002; Sun et al., 2003].

Because raft domains are known to be involved in cell signaling in caveolin-negative cells [Magee et al., 2002; Horejsi, 2003; Saeki et al., 2003], signal transduction through rafts in cancer may be caveolin-independent. Down-regulation of caveolins is a common characteristic of malignant cells [Wiechen et al., 2001];

I. Reservoir for signaling molecules.

(1) Maintenance of signaling molecules in an inactivated state.

(2) Sequestration of signaling proteins away from phosphatases.

(3) Sequestration of GPI-anchored proteins.

(4) Allows for increased collision kinetics for dimerization of receptors (e.g. EGFR, PDGFR)

II. Rapid response to external stimuli.

(5) Pre-assembled signaling complexes for rapid receptor signaling.

(6) Cross-talk between signaling molecules (e.g. kinases & G-proteins; protein-protein interactions).

III. Intracellular trafficking of the signaling molecules.

(7) Internalization of the receptor / ligand complex.

(8) Receptor recycling.

IV. Trafficking within the membrane.

(9) Membrane partitioning of signaling proteins.

(10) Sequestration and transport of cholesterol.

V. Organization of signaling pathways.

● Exclusion of signaling molecules from rafts.

● Recruitment of cytosolic proteins.

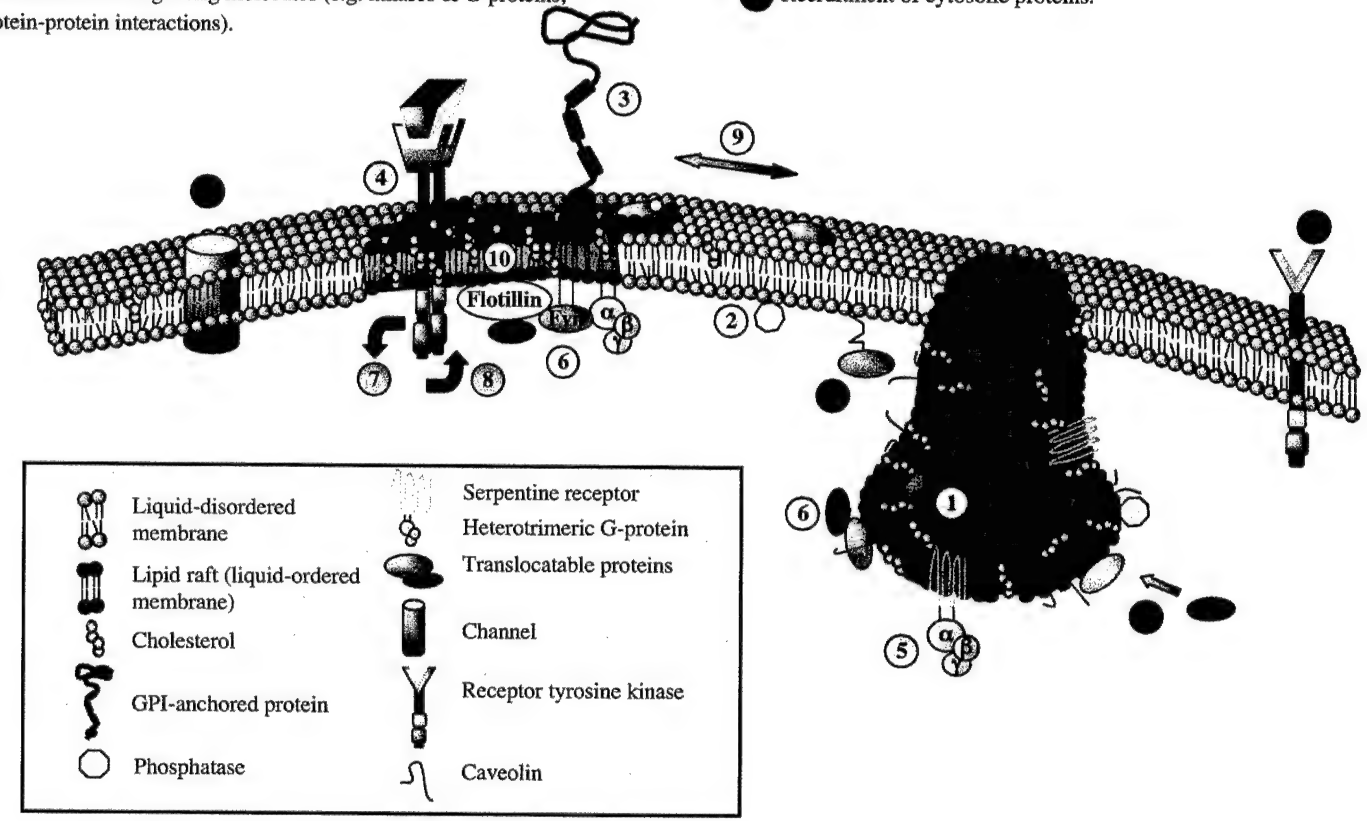

Fig. 2. Caveolae and flat lipid rafts. The diagram illustrates known and hypothetical mechanisms for control of signal transduction by caveolar and non-caveolar rafts. It is not intended that most of the mechanisms attributed to caveolae cannot be mediated by flat rafts, and vice versa.

consequently, despite the apparent connection between caveolin-1 and PCa progression, it is important to realize that, in cancer cells, rafts might not require caveolins for the performance of signaling functions relevant to tumor progression. The reader is directed to several recent reviews for in-depth discussions of raft-dependent signal transduction mechanisms [Simons and Toomre, 2000; Galbiati et al., 2001b; Magee et al., 2002; Zajchowski and Robbins, 2002]. Our own view is that in cancer cells, caveolar and non-caveolar rafts may be equally important in sequestering signaling molecules and/or for signal processing necessary for cancer cell growth and survival in the face of apoptotic triggers present in the tumor environment.

Addition of exogenous LDL to cultures of PC-3 human PCa cells has been reported to stimulate cell growth [Hughes-Fulford et al., 2001]. At least one older study reported that addition of LDL to cultured cells was sufficient to "transform" them, using in vitro criteria [Zwijsen, 1992]. These results are intriguing when one considers the aforementioned literature demonstrating that solid tumors can accumulate cholesterol. How might we interpret these findings in the context of lipid raft-mediated signaling? Cholesterol has been demonstrated in a large number of studies to be a lipid raft component that is essential for the functional integrity of caveolar and non-caveolar rafts. This literature persuasively illustrates that raft-dependent signaling events can be inhibited by dispersing cholesterol or removing it from the membrane with cholesterol-binding compounds [Liu et al., 1997a; Pike and Miller, 1998; Peiro et al., 2000; Parpal et al., 2001]. On the other hand, experiments with artificial membranes have demonstrated that liquid-ordered, sphingomyelin-enriched lipid microdomains can exist in the absence of cholesterol [Milhiet et al., 2002]. These cholesterol-poor rafts can actually be disrupted by cholesterol addition [Milhiet et al., 2002]. Collectively, this information suggests that lipid microdomains in living cells might be heterogeneous in structure and function and might respond in a variety of ways to changes in steady-state cholesterol levels in the membrane. Thus, from first principles one can conclude that the accumulation of cholesterol that can occur in tumors, in concert with other tumor-associated alterations in normal mechanisms of cholesterol homeostasis, is likely to alter raft-dependent signaling in tumor cells. But which signal transduction mechanisms might be altered by changes in cholesterol metabolism in tumors and how might they be affected by these changes?

The possibility that some signaling mechanisms relevant to PCa cancer progression may be dependent on cholesterol present in the plasma membrane is currently under study in our laboratories. Zhuang et al. [2002] recently demonstrated that LNCaP androgen-responsive human PCa cells can be stimulated to undergo apoptosis in response to treatment with filipin, a polyene macrolide that binds cholesterol and disperses it in the plane of the membrane. That study showed that signaling through the Akt serine–threonine kinase is partly dependent on the integrity of plasma membrane rafts and that the effects of filipin on Akt signaling and apoptosis can be attenuated by repletion of the membrane with cholesterol. Akt is an important node for cell survival and growth signals in PCa and in other solid tumors [Paez and Sellers, 2003]. Akt is also believed to be physiologically relevant to clinical PCa because PTEN, a lipid phosphatase that is an important negative regulator of this pathway, is inactivated in a significant fraction of aggressive PCa [McMenamin et al., 1999]. In another study that we have recently submitted for publication, Zhuang, Kim and colleagues go on to show that this dependence on lipid rafts for ligand-activated signaling through Akt extends even to normal prostate epithelial cells (PrEC). Unlike the situation in LNCaP cells, cholesterol-binding agents did not stimulate apoptosis in PrEC, indicating that the cancer cells may have become dependent on a cholesterol-mediated cell survival pathway. Interestingly, the Zhuang, Kim et al. study (unpublished results) also showed that simvastatin, which has been demonstrated previously to stimulate apoptosis in cancer cells, also inhibits Akt signaling in LNCaP cells. Furthermore, the cholesterol content of lipid rafts in these cells was shown to be dramatically decreased with simvastatin treatment. This result indicates that it may be possible to target raft-dependent cell survival mechanisms in PCa cells by pharmacologic intervention using FDA-approved drugs that have been demonstrated in clinical trials to be well tolerated with long-term therapy. The finding that cholesterol-binding polyene macrolides can be potent stimulators of cancer cell death and that, in contrast, normal cells are

relatively resistant to this treatment, was actually demonstrated and reported over 25 years ago [Fisher et al., 1975]. This was before the phenomenon of apoptosis as we know it today was established as a cellular process, although these older data are consistent with our own studies of cholesterol-dependent survival mechanisms in prostatic cells.

Several other groups have recently reported findings in other cell systems consistent with a role for rafts in signal transduction through Akt [Bauer et al., 2003; Podar et al., 2003]. Interestingly, the Hemmings laboratory has identified an enzymatic activity capable of phosphorylating Akt on Ser-473 as a protein that resides in the lipid raft subcellular compartment [Hill et al., 2002]. The findings reported in that study are significant because, although it is known that translocation to the plasma membrane from the cytosol is a feature of Akt phosphorylation by upstream activators, there is still considerable controversy about the mechanism by which Akt becomes phosphorylated on its two principal regulatory sites (Thr-308 and Ser-473) [Scheid and Woodgett, 2003]. The presence of an Akt kinase in the raft compartment suggests that cells may employ raft microdomains as a means to rapidly mobilize or, alternatively, repress the enzymatic or binding functions of the molecule.

In a third study from our laboratories, Kim and colleagues demonstrated that signaling to the transcription factor, STAT3, by IL-6 also involves lipid rafts in LNCaP cells [Kim et al., 2003]. Increases in circulating IL-6 are associated with PCa progression [Nakashima et al., 2000], and IL-6 has been shown in cell culture models to be an inducer of neuroendocrine characteristics in PCa cells [Deeble et al., 2001]. Neuroendocrine properties in prostate and other solid tumors, and in animal models of PCa, have been associated with more aggressive disease [Abrahamsson, 1999]. Steady-state increases in STAT3 activation are also associated with advanced PCa [Mora et al., 2002]. In our study by Kim et al., IL-6 induced phosphorylation of STAT3, its translocation from the cytoplasm to the nucleus, as well as promoter activity of the neuroendocrine marker, neuron specific enolase (NSE) and accumulation of NSE protein, were partly dependent on intact plasma membrane rafts. Phosphorylated STAT3 also predominantly localized to the raft compartment after stimulation of the cells with IL-6. Consequently, these findings represent another demonstration of a cholesterol-dependent signal transduction mechanism underlying a process that is potentially relevant to disease progression in humans. Interestingly, neuroendocrine differentiation in PCa cells most likely occurs independently of androgenic signaling [Adam et al., 2002], suggesting the possibility that raft-dependent signals may operate promiscuously (i.e., without the influence of androgen) in hormone-refractory disease and in the androgen-depleted state. This hypothesis is potentially all the more relevant because of the demonstration in animals [Cinci et al., 1993] and humans [Moorjani et al., 1988] that androgen suppression can induce hypercholesterolemia.

A MODEL INTEGRATING THE PHENOMENON OF INCREASED MEMBRANE CHOLESTEROL IN TUMOR CELLS WITH LIPID RAFT SIGNALING

Inspection of Figure 2 makes it abundantly clear that both caveolar and non-caveolar rafts might alter signal transduction processes in cancer cells in a multitude of ways. Is there a simpler model that would allow testing of the hypothesis that elevation of cholesterol content in tumor cell membranes promotes disease progression? If so, how might this model be applied toward the identification of new targets for disease therapy? In imagining such a model, it is useful to understand how the concept of the lipid microdomain explains the phenomenon of signal transmission by GPI-anchored proteins. It is now well established that GPI-anchored proteins reside in lipid rafts along with an array of other signal transducing molecules. Although in isolation individual GPI-anchored proteins do not appear capable of generating signals, when cross-linked by antibodies they are able to generate many different types of signals, including Ca^{2+} mobilization, inositol phosphate production, as well as a range of cellular responses such as proliferation, growth factor production and apoptosis. It is reasonable to speculate that what is occurring when the GPI-anchored proteins are cross-linked is that as the proteins are being pulled together, their associated raft domains are also being brought together as well. Thus, the small, isolated rafts coalesce to form substantially larger rafts (think: island). This concept is illustrated in Figure 3. Isolated rafts are likely to be relatively small, with limited compositional complexity.

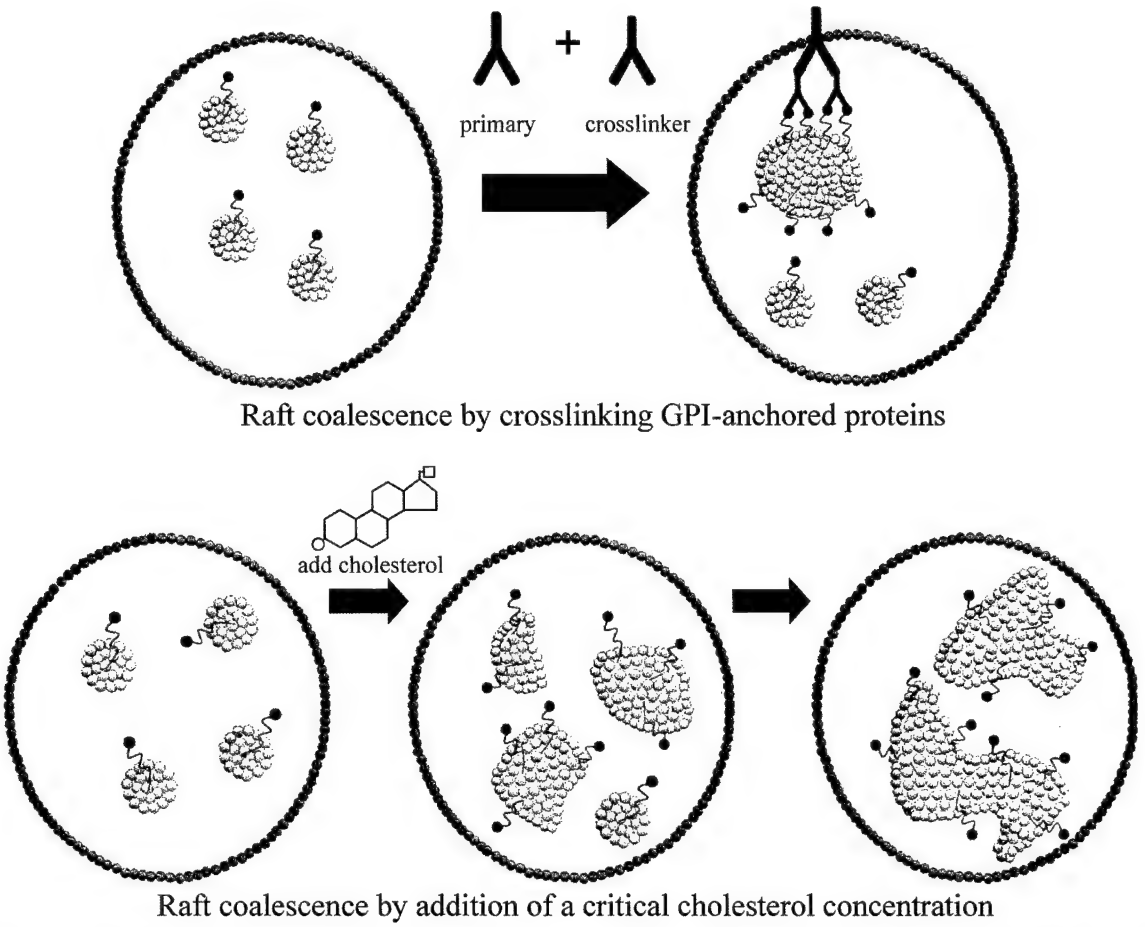

Fig. 3. A model for how increases in membrane cholesterol might alter signal transduction in cancer cells. Cross-linking of GPI-anchored proteins may induce the coalescence of lipid rafts, thereby activating signaling mechanisms. Similarly, increases in membrane cholesterol beyond some critical concentration may coalesce rafts, thereby sequestering oncogenic signaling molecules within rafts, increasing compositional complexity of individual rafts, and excluding negative regulators from the raft compartment.

As discussed above, although a variety of signaling molecules are found in rafts, it is probable that not all rafts are compositionally equivalent. Consequently, the process of raft cross-linking would not only create large rafts, but would probably increase raft complexity by assembling rafts with varying protein composition. In addition, because large, coalesced rafts maintain the same surface area as the sum of all the isolated rafts, but have a dramatically decreased circumferential length, fewer raft proteins will be present at the raft/non-raft (liquid-disordered) interface. Therefore, fewer raft proteins would be available to be regulated by moieties that are excluded from rafts but which may be abundant in the membrane-at-large. Solomon and co-workers have hypothesized for almost a decade that coalescence of rafts is the underlying mechanism for signaling induced by antibody cross-linking of GPI-anchored proteins [Solomon, 1996].

How does the cross-linking hypothesis apply to the observation that increases in cholesterol in tumor cell membranes, either from dietary or other factors, may promote PCa growth and disease progression? We know from experiments with model membranes that moderate increases in the level of membrane cholesterol (10–20%) reduces the number of isolated rafts and causes the formation of larger rafts [Lawrence et al., 2003]. Consequently, the literature already provides support for the idea that as membrane cholesterol levels increase, larger raft structures, with a smaller total perimeter, begin to form. The model illustrated in Figure 3 illustrates that raising cholesterol levels beyond some critical concentration may result in the coalescence of smaller raft domains, analogous

to the manner by which rafts might coalesce in response to cross-linking of GPI-anchored proteins. This may serve to sequester, and thereby stimulate, "on" signals to oncogenic pathways, as well as exclude negative regulators that contribute "off" signals in the normal environment. This model is consistent with the reported association between caveolin-1 expression and PCa progression [Yang et al., 1998] in the sense that higher caveolin levels may reflect an expansion of the raft compartment in aggressive tumor cells.

Importantly, the model illustrated in Figure 3 can be tested empirically. In unpublished studies from our group, Zhuang, Kim and colleagues have demonstrated that raising serum cholesterol in SCID mice harboring LNCaP PCa xenograft tumors results in an increase in cholesterol in lipid raft membranes. Importantly, this increase in raft cholesterol content was shown to correlate with alterations in several indices of oncogenic signal transduction, including an increase in the levels of raft proteins phosphorylated on tyrosine, an increase in phosphorylated Akt, and a decrease in apoptotic rates as evaluated by TUNEL.

CONCLUDING REMARKS

Cholesterol accumulation in PCa cells, in concert with alterations in cholesterol metabolism associated with age and malignancy in the prostate, is likely to alter signal transduction mechanisms underlying PCa progression in profound ways. We have proposed that one possible consequence of progressive increases in membrane cholesterol is the expansion of the tumor cell lipid raft compartment, a change in the plasma membrane that may potentiate oncogenic pathways of cell signaling. The ability to isolate lipid rafts from cells and tumors using established biochemical methods allows for raft proteins that respond to specific signals, such as soluble factors that promote cancer cell growth and survival, to be identified and characterized. Large-scale cataloging of lipid raft proteins using mass spectrometry is now ongoing by a number of groups [Bini et al., 2003; Foster et al., 2003]. This will allow the direct testing of the hypothesis diagrammed in Figure 3, as well as the identification of signaling proteins that may associate with rafts stably or transiently during the multiple processes illustrated in Figure 2. The identification of these raft-associated proteins, in combination with experiments designed to understand the functional implications of their association with cholesterol-rich membrane domains, will provide new insight into signal transduction processes related to cancer spread. We believe they will also provide a wealth of new targets for cancer therapy and possibly new biomarkers that will be useful in a clinical setting.

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Involvement of Cholesterol-Rich Lipid Rafts in Interleukin-6-Induced Neuroendocrine Differentiation of LNCaP Prostate Cancer Cells

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IL-6 is an inflammatory cytokine that has been linked to aggressive prostate cancer (PCa). Previous studies have demonstrated that IL-6 can enhance the differentiation of PCa cells toward a neuroendocrine (NE) phenotype, a possible indicator of hormone-refractory disease. In this report, we present evidence that the mechanism of IL-6-stimulated NE differentiation employs a detergent-resistant (lipid raft) membrane compartment for signal transduction in LNCaP PCa cells. Signal transducer and activator of transcription (STAT)3, a mediator of IL-6 signaling, was rapidly phosphorylated and translocated to the nucleus in LNCaP cells treated with IL-6. Both processes were inhibited by filipin, a cholesterol-binding compound that disrupts plasma membrane lipid rafts. Isolation of Triton X-100-insoluble raft fractions from LNCaP cells by discontinuous sucrose gradient centrifugation demonstrated that the 80-kDa IL-6 receptor localized almost exclusively to the raft compartment. Although STAT3 was located predominantly in the Triton X-100-soluble subcellular fraction in exponentially growing cells, abundant phosphorylated STAT3 was detected in the raft fraction after stimulation with IL-6. Increases in expression of the NE marker, neuron-specific enolase, and neuron-specific enolase promoter activity after IL-6 treatment were reduced after membrane rafts were disrupted by filipin treatment. LNCaP cells expressed the raft-resident proteins flotillin-2 and $G_{i\alpha 2}$, but notably not caveolins, the predominant structural protein present in caveolar membrane rafts in many tissues and tumor cells. These results are the first to define a role for lipid raft membrane microdomains in signal transduction mechanisms capable of promoting the NE phenotype in PCa cells, and they demonstrate that the raft compartment is capable of mediating such signals in the absence of caveolins. Our results also suggest a mechanistic role for membrane cholesterol in cell signaling events relevant to PCa progression. (*Endocrinology* 145: 613–619, 2004)

NEUROENDOCRINE (NE) PROPERTIES expressed by tumor cells can indicate poor prognosis or the likelihood of the presence of aggressive disease in a variety of solid tumor types, including prostate cancer (PCa) (1, 2). Tumor cells that express NE characteristics can be postmitotic (3); however, they typically produce cytokines capable of stimulating growth and survival of neighboring adenocarcinoma cells in a paracrine manner (4, 5). In addition, evidence that tumor cells exhibiting NE-like properties can remain capable of proliferation has been obtained in cell culture models (6) and with *in vivo* model systems (7). These findings indicate that tumor progression coinciding with NE differentiation can result in the emergence of an inherently more aggressive tumor cell population.

The molecular mechanisms by which PCa cells acquire an NE phenotype are incompletely understood; however, evidence indicates that this process involves the action of one or more of several possible signal transduction pathways. Previous studies have used the human LNCaP cell line to study the manner in which human PCa cells acquire features of NE differentiation (2, 3, 6, 8–10). LNCaP cells express a marginal NE phenotype under basal conditions, but NE characteristics are substantially enhanced in this cell line by certain culture manipulations, including prolonged androgen depletion (11), pharmacological elevation of intracellular cAMP (12), and exposure of cells to the epidermal growth factor receptor tyrosine kinase (ErbB family) ligand heparin-binding epidermal growth factor-like growth factor (HB-EGF) (6, 13) or to the cytokine IL-6 (3). The majority of published reports have focused on IL-6 as the primary inducer of this phenotype, in part, because elevated circulating IL-6 levels are associated with aggressive PCa and may be an indicator of hormone-refractory disease (14–16). NE differentiation in LNCaP and other cell lines can be evoked as a result of the activation of multiple, sometimes independent, signal transduction mechanisms. For example, in LNCaP cells, IL-6 triggers phosphorylation and nuclear import of the transcription factor, signal transducer and activator of transcription (STAT)3 and induces cell cycle arrest (10, 17), whereas HB-EGF induces NE differentiation by a STAT3-independent, ERK-MAPK-dependent process that coincides with continued cell cycle transit (6).

STATs are well known as cytosolic proteins that are recruited to the plasma membrane by members of the JAK family of tyrosine kinases (18). After activation, STATs dimerize through SH2-phosphotyrosyl interactions and migrate to the nucleus, where they are incorporated into transcriptional complexes (18–20). The manner in which STATs

Abbreviations: HB-EGF, Heparin-binding epidermal growth factor-like growth factor; IL-6R, IL-6 receptor; NE, neuroendocrine; NSE, neuron-specific enolase; OCG, octylglucoside; PCa, prostate cancer (prostate adenocarcinoma); PMSF, phenylmethylsulfonylfluoride; STAT, signal transducer and activator of transcription.

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are sequestered before activation is still incompletely understood. Recent studies have indicated that a significant fraction of latent and active STATs reside in detergent-resistant membrane domains generally referred to as lipid rafts (21, 22). Lipid rafts are liquid-ordered membrane assemblies that are rich in cholesterol and glycosphingolipids relative to the substantially more abundant liquid-disordered phase of the plasma membrane (23). Lipid rafts sequester signaling proteins of many kinds, including heterotrimeric G protein subunits, receptor tyrosine kinases, and Src-like kinases. Although their biological function is still poorly understood, rafts have been shown to act as membrane platforms for regulating signal transduction in many cell types, including tumor cells (24).

The potential relevance of lipid raft membrane domains to PCa was first suggested by observations that caveolin-1, a raft-resident structural protein thought capable of directly regulating a variety of signal transduction molecules, is a marker of aggressive disease in PCa and other cancers (25–29). Caveolin-1 is capable of promoting hormone-mediated tumor cell survival and metastatic dissemination in model systems of PCa (26). Furthermore, caveolin-1 may be a mediator of androgenic signaling by a mechanism involving a direct protein-protein interaction with the androgen receptor (30). Lipid rafts also seem to be capable of processing signals relevant to tumor progression even in the absence of caveolin-1. Regulation of the Akt/protein kinase B pathway and cell survival signaling by a cholesterol-dependent mechanism involving lipid rafts was also recently demonstrated in caveolin-negative LNCaP cells (31).

In this study, we present evidence that signal transduction through lipid rafts is involved in IL-6- and STAT3-mediated induction of NE properties in LNCaP cells. These results support a role for lipid raft microdomains as essential mediators of disease progression in PCa. They also suggest that the presence of caveolins in the raft membrane compartment is not necessary for transmission of certain cellular signals involved in the promotion of aggressive disease.

Materials and Methods

Reagents

Human recombinant IL-6 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies against caveolin-1, caveolin-2, pan-caveolin, flotillin-2, and neuron-specific enolase (NSE) were purchased from BD Biosciences (San Diego, CA). Anti-phospho-STAT3 (Tyr705) and anti-STAT3 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Reagents for sucrose gradient ultracentrifugation were the highest possible grade and were obtained from Sigma (St. Louis, MO). Antibodies against IL-6 receptor (IL-6R), and $G_{i\alpha 2}$ were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against NSE were from Neomarkers (Fremont, CA). The Micro BCA protein assay kit was used for protein measurement (Pierce Chemical Co., Rockford, IL). Polyfect transfection reagents were purchased from Qiagen (Valencia, CA). Infinity cholesterol determination reagents and filipin were obtained from Sigma.

Cultured cells

The human PCa cell line LNCaP and PC3 were purchased from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 or DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc., Grand Island, NY). These supplements were used in all media unless otherwise indicated. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 C.

Sucrose gradient ultracentrifugation

LNCaP cells were lysed in 1% Triton X-100 in 25 mM 2-(*N*-morpholino)-ethanesulfonic acid, 150 mM NaCl (pH 6.5), 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonylfluoride (PMSF) followed by mechanical disruption with eight strokes of a Dounce homogenizer. Cell lysates were diluted 1:1 with 60% sucrose (final sucrose concentration of 30%) and layered on a 40% sucrose cushion, followed by successive 2-ml additions of 25%, 20%, 15%, 10%, 5%, 0% (20 mM KCl) sucrose solution. Ultracentrifugation was performed at 100,000 × *g* for 20 h in a Beckman SW28 rotor (Beckman Coulter, Fullerton, CA). All experimental steps were performed on ice or at 4 C.

Lipid raft isolation by successive detergent extraction

Extraction of Triton-soluble and -insoluble membrane components was performed as described (32). Briefly, LNCaP cell lysates were prepared in buffer A [25 mM 2-(*N*-morpholino)-ethanesulfonic acid, 150 mM NaCl (pH 6.5)], and an equal volume of the same buffer with 2% Triton X-100, 2 mM Na_3VO_4, and 2 mM PMSF was added. After 30 min of incubation, lysates were centrifuged, and supernatants (containing the Triton-soluble fraction) were removed. Insoluble pellets were resuspended with buffer B [1% Triton X-100, 10 mM Tris-Cl (pH 7.6), 500 mM NaCl, 2 mM Na_3VO_4, 60 mM β-octylglucoside, and 1 mM PMSF] for 30 min on ice. Triton-insoluble and octylglucoside (OCG)-soluble supernatants were collected after 20 min of centrifugation at 15,000 × *g*.

Cholesterol measurements

Cholesterol determinations were performed on 300-μl fractions harvested from sucrose gradients described above or in total cell membranes prepared by resuspending cells in a hypotonic buffer [50 mM HEPES, pH 7.4; 10 mM NaCl; 5 mM $MgCl_2$; 0.1 mM EDTA; and protease inhibitors] followed by mechanical disruption (12 strokes with a Dounce homogenizer) and centrifugation (9,000 × *g* for 10 min). Lipids were solubilized in chloroform, extracted two times through H_2O, dried, and subjected to cholesterol determination using the Infinity cholesterol determination assay kit (Sigma).

Lipid raft disassembly

Lipid raft disruption was accomplished by treating cells with filipin, a cholesterol-binding polyene macrolide that has been shown to disassemble lipid rafts by dispersing cholesterol in the membrane, thereby interfering with transmission of raft-dependent signals (24, 33, 34). Cells were treated with varying concentrations of filipin (see figures) at 37 C in serum-free culture medium before assay.

Western blot analysis

Proteins isolated from cell fractions as described above were subjected to SDS-PAGE and electroblotted onto nitrocellulose. Blots were stained with Ponceau S to verify uniform transfer and equal protein loading (where appropriate) and subjected to immunoblotting with various antibodies as previously described (6).

Indirect immunofluorescence cell staining

Low-density LNCaP cells were serum starved for 12 h, followed by challenge with 100 ng/ml IL-6 for 15 min. In some cases, cells were pretreated with 2 μg/ml filipin before IL-6 treatment. Cells were fixed with ice-cold methanol and incubated with anti-phospho-STAT3 antibody in 2% BSA solution, followed by secondary antibody conjugated to fluorescein isothiocyanate. After mounting and incubation with 4′-containing mounting agent, cells were analyzed by fluorescence microscopy.

Construction of NSE-luciferase promoter reporter plasmid and measurement of promoter activity

A fragment of approximately 1.3-kb encoding the NSE promoter was amplified from genomic DNA (Promega Corporation, Inc., Madison,

WI) using the Advantage-GC Genomic PCR reagent (BD Biosciences Clontech, Palo Alto, CA) and the primers: 5′-GCGGCTAGCTGTATGCAGCTGGACCTAGGAGAGAAGCAG-3′ and 5′-GCGAGATCTCGGTGGTAGTGGCGGTGGCGGTGGCGGTGG-3′. The primers incorporated restriction sites for NheI and BglII, respectively (*underlined*). The PCR product was digested with NheI and BglII and subcloned into the reporter vector, pGL3-Basic (Promega Corporation Inc.) to generate pNSE-Luc. The integrity of the construct was confirmed by DNA sequencing. NSE promoter activity was determined after transient transfection with the NSE promoter-luciferase reporter plasmid, using the Polyfect reagent. Experiments using filipin and IL-6 were performed 24 h after transfection. At the end of treatment, medium was removed, and cells were lysed with 100 μl reporter lysis buffer. Lysates were prepared by freeze-thaw (three times), and insoluble material was pelleted with high-speed centrifugation. Luciferase activity was normalized to β-Gal activity.

Statistical analysis

Experimental data were compared using Student's two-tailed *t* test.

Results

To determine the role of lipid raft microdomains in IL-6 signaling independently of the contribution of caveolin proteins, we used LNCaP cells that do not express detectable levels of caveolins. The results shown in Fig. 1 demonstrate that LNCaP cells contain a verifiable lipid raft membrane fraction that does not contain caveolins, consistent with our previous report (31). In the experiment shown, this detergent-resistant membrane fraction contained the raft protein markers, flotillin-2 and $G_{i\alpha 2}$, but did not contain detectable caveolin-1 and -2, as demonstrated by immunoblotting with antibodies to caveolin-1 and -2 or with a pan-caveolin antibody (Fig. 1). In contrast to LNCaP cells, the more aggressive PC-3 PCa cell line expressed caveolin-1 and -2, which partitioned, as expected, into the raft fraction obtained from these cells. The caveolin-negative LNCaP cells used for the experiments shown in Fig. 1 were used throughout these studies.

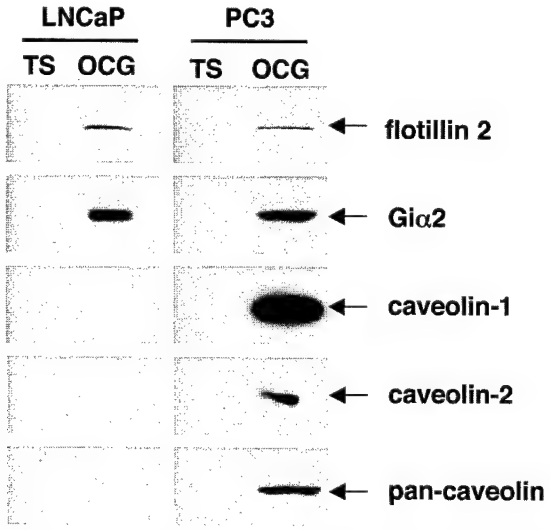

FIG. 1. LNCaP PCa cells are caveolin-negative. LNCaP and PC3 cells in serum-containing medium were harvested, and cell lysates were separated into Triton-soluble (TS) and Triton-insoluble/OCG-soluble fractions. Localization of flotillin-2, $G_{i\alpha 2}$, and caveolin isoforms was determined by Western blot. The OCG fraction is the detergent-resistant membrane (lipid raft) fraction.

Lipid raft disruption inhibits IL-6-mediated STAT3 phosphorylation and nuclear translocation

IL-6 stimulates NE differentiation in PCa cell lines through a STAT3-dependent pathway (10). To study the possible functional contribution of plasma membrane rafts to expression of the NE phenotype, we disrupted the rafts (see *Materials and Methods*) before treatment of LNCaP cells with IL-6. Raft disruption with the cholesterol-binding compound, filipin, blocked STAT3 phosphorylation induced by 100 ng/ml IL-6 (Fig. 2A), indicating that STAT3 activation is a raft-dependent event. Immunofluorescence cell staining with an anti-phospho-STAT3 antibody demonstrated an increase in STAT3 phosphorylation and migration of phosphorylated STAT3 to the nucleus after IL-6 treatment (Fig. 2B, *middle*). These events were inhibited when the rafts were disrupted (Fig. 2B *right*).

IL-6R is associated with lipid rafts in replicating LNCaP cells

To investigate the localization of STAT3 and the 80-kDa ligand-binding IL-6R in LNCaP cells under exponentially growing conditions, we evaluated the buoyant density of these proteins in discontinuous sucrose gradients. Cells growing at subconfluent density in serum (in the absence of IL-6) were solubilized with Triton X-100-containing lysis buffer, homogenized, and resuspended in the same volume of 60% buffered sucrose solution. Samples were loaded onto step gradients containing 0–40% sucrose. After ultracentrifugation (20 h), fractions were removed sequentially from the top of the gradient and examined by immunoblot and cho-

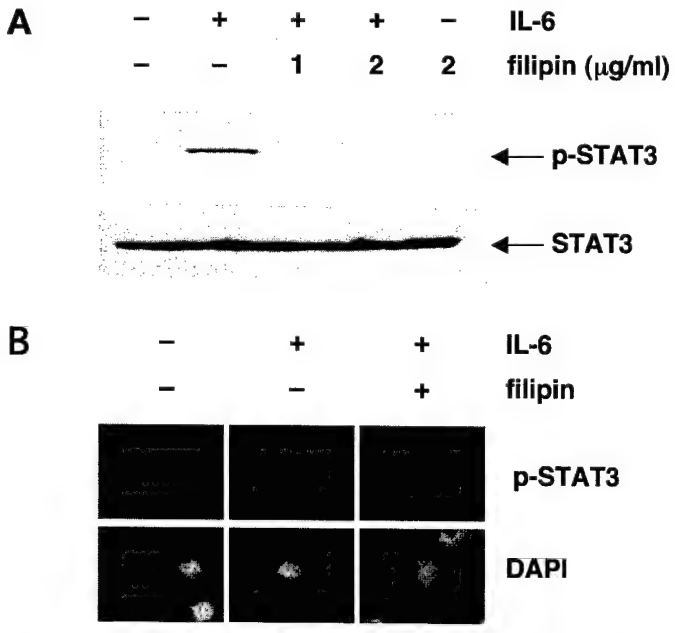

FIG. 2. Disruption of plasma membrane rafts inhibits IL-6-induced STAT3 phosphorylation and nuclear translocation. Quiescent LNCaP cells were preincubated with filipin at the indicated concentrations for 1 h, followed by treatment with IL-6 for 15 min. A, Phosphorylation of STAT3 determined by Western blot. B, Phosphorylated STAT3 observed by immunofluorescence staining using anti-p-STAT3 antibody. Nuclei were counterstained with 4′,65-diamidino-2-phenylindole.

lesterol measurement. Light buoyant density fractions that contained the majority of the raft-associated proteins, flotillin-2 and $G_{i\alpha 2}$, were identified and were verified to contain high levels of cholesterol (Fig. 3), indicating that these fractions correspond to lipid raft membranes. In the experiment shown in Fig. 3, fraction 11 contained essentially all of the raft protein $G_{i\alpha 2}$, the majority of flotillin-2, and the highest levels of cholesterol. This fraction also contained essentially all of the detectable IL-6R, indicating that the IL-6R localizes exclusively to the lipid raft membrane compartment in LNCaP cells under exponential growing conditions. STAT3 was also detected in fraction 11, although the majority of STAT3 was present in the heavy fractions.

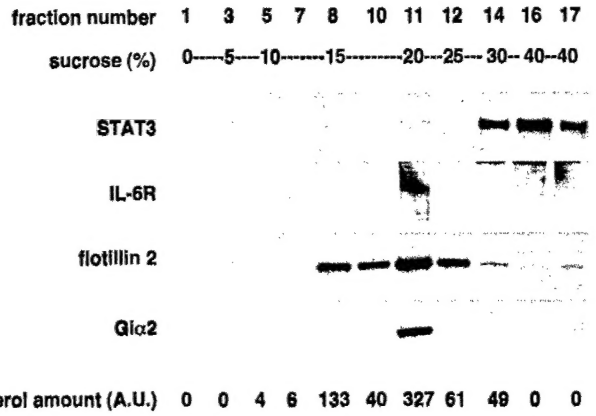

FIG. 3. Lipid rafts in LNCaP cells contain IL-6R and STAT3. Exponentially growing LNCaP cells (in the absence of IL-6) were lysed in Triton-containing buffer and prepared for sucrose gradient ultracentrifugation as described in *Materials and Methods*. Expression of STAT3, IL-6R, flotillin-2, and $G_{i\alpha 2}$ were analyzed by Western blot. Relative cholesterol levels in each fraction are shown *below the lower panel*. A.U., Arbitrary units.

Phosphorylated STAT3 accumulates in the lipid raft fraction after IL-6 stimulation

To investigate whether IL-6 employs lipid raft microdomains to transmit a cellular signal, the subcellular location of phosphorylated STAT3 was evaluated after IL-6 treatment. The majority of phosphorylated STAT3 was detected in the $G_{i\alpha 2}$-enriched fraction, 15 min after stimulation with IL-6, as evaluated by sucrose density centrifugation and immunoblot (Fig. 4A). A similar result was observed using a separate method that employs differences in detergent solubility to isolate raft membranes (32) (Fig. 4B). These results indicate that IL-6 signaling to STAT3 occurs through a lipid raft membrane domain. Interestingly, the data shown in Fig. 4B also indicate that phosphorylated STAT3 molecules (*i.e.* actively signaling molecules) are relatively enriched in the raft fraction, in comparison with the nonraft subcellular compartments, where the majority of STAT3 resides (consistent with the data shown in Fig. 3).

Lipid raft disruption antagonizes IL-6-mediated stimulation of NE properties

IL-6 treatment stimulates an enhancement of NE properties in LNCaP cells (3). To determine whether this effect is mediated by lipid rafts, cells in which the rafts were disrupted were compared with raft-competent control cells, with respect to the expression of NE properties after IL-6 treatment under the conditions used for the experiment shown in Fig. 2. Expression of the NE marker NSE increased about 6-fold with IL-6 treatment at 5 d, an effect significantly inhibited when rafts were disrupted with filipin (Fig. 5A). The inhibitory effect of filipin on IL-6 expression was dose dependent. A similar effect was demonstrated at the transcriptional level when luciferase activity produced from a transiently transfected NSE promoter-reporter construct was

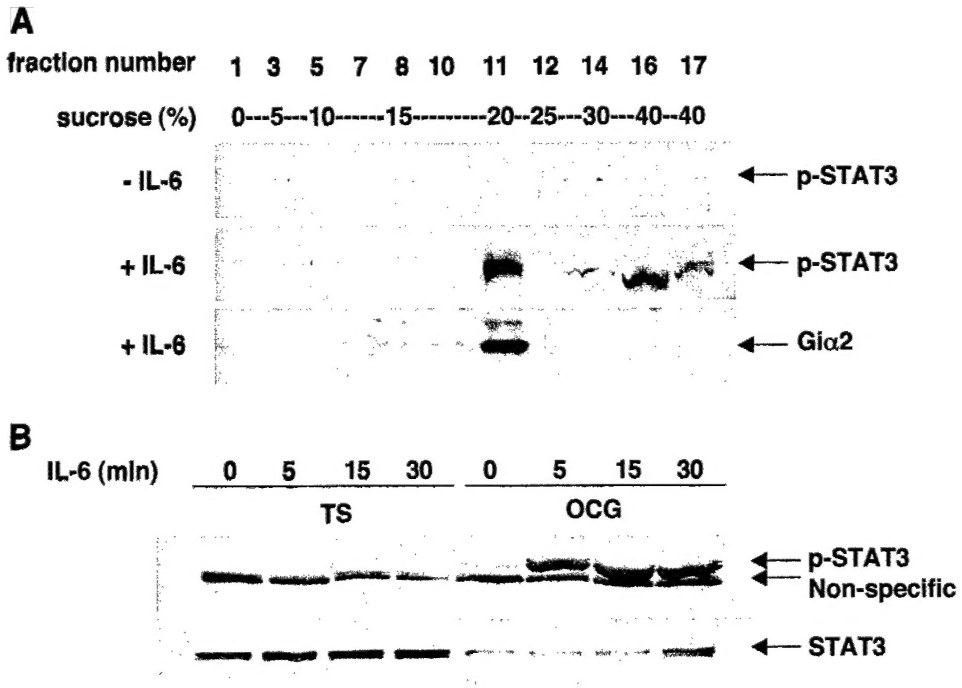

FIG. 4. Lipid rafts in LNCaP cells are enriched in phosphorylated STAT3 after treatment with IL-6. Quiescent cells were stimulated with IL-6 in serum-free medium. A, After 15 min, LNCaP cell lysates from vehicle (−IL-6) and IL-6-treated (+IL-6) conditions were prepared for sucrose gradient ultracentrifugation. p-STAT3 and $G_{i\alpha 2}$ were detected by Western blot. B, At the indicated times, IL-6-treated LNCaP cells were harvested, and lysates were separated into TS and Triton-insoluble/OCG-soluble (OCG) fractions. Phosphorylated and total STAT3 were detected by Western blot.

analyzed (Fig. 5B). These findings indicate that IL-6 stimulation of NE properties is raft-dependent. Consistent with this conclusion, addition of cholesterol-cyclodextrin complexes to the cell cultures, a procedure that increases levels of membrane cholesterol and the size of the raft compartment, stimulated NSE promoter activity about 20% in the absence of IL-6 (data not shown).

Discussion

In this study, we have identified a novel role for detergent-resistant plasma membrane rafts in IL-6-mediated promotion of NE differentiation in LNCaP PCa cells. IL-6, a cytokine that has been linked to human PCa progression (15), has been identified previously as being capable of enhancing the NE characteristics of LNCaP and other PCa cell lines (3, 10). The evidence we present in support of a role for lipid rafts in NE differentiation in LNCaP cells is the following: 1) raft disruption by dispersion of cholesterol in the plasma membrane inhibited STAT3 phosphorylation and translocation of STAT3 to the nucleus in LNCaP cells treated with IL-6; 2) the 80-kDa IL-6R localized essentially entirely (and STAT3 localized partially) to a lipid raft compartment in LNCaP cells under exponential growth conditions; 3) the majority of activated STAT3 localized to the raft compartment in response to treatment of cells with IL-6; and 4) raft disruption inhibited NSE protein accumulation, and induction of NSE promoter activity, in response to IL-6. Our findings provide the first evidence that the IL-6 signaling mechanism employs a cholesterol-rich membrane raft compartment for transmission of the cytokine-mediated signal in PCa cells. The LNCaP cell line is used widely for *in vitro* and *in vivo* studies of PCa because it expresses a range of properties characteristic of differentiated human prostate epithelial cells (*e.g.* prostate-specific antigen). LNCaP cells respond to androgen by modulating growth and altering gene expression. Consequently, these studies may be relevant to mechanisms of NE differentiation *in vivo*, where NE properties in prostate tumors have been associated with aggressive or hormone-refractory disease (1). Although the role of plasma membrane events in the activation of STATs is still poorly understood, our results are consistent with recent reports that STATs employ raft-dependent signaling mechanisms in other cell types (22, 35). An important distinction, however, is that these previous studies employed cells that express the raft-resident protein, caveolin-1. In contrast, our experiments have employed caveolin-negative cells, thereby demonstrating that caveolins are not an obligatory component of the STAT3-mediated pathway described here.

Although the IL-6/STAT3/NE differentiation mechanism we focus on in the present study is independent of contributions from caveolins, our findings are consistent with previous reports implicating caveolin-1 in PCa progression (26–28). This is because caveolins localize essentially exclusively to lipid raft membranes, an observation that points to the membrane raft compartment as an important element of the mechanism by which PCa cells survive in the host and progress to a metastatic state. The present study and earlier studies, reporting increases in caveolin-1 expression in aggressive PCa, point to a role for the plasma membrane raft compartment as a potential locus of cell signaling events relevant to PCa progression. Caveolin-1 has been reported to modulate the activity of a number of signaling molecules capable of transiting caveolar lipid rafts, in some cases by direct protein-protein interaction with caveolin proteins (30, 36–38). Thus, the cholesterol- and sphingolipid-rich raft fraction may serve as an important node for signal transduction events regulating PCa cell growth and survival. Consistent with this idea, our laboratory has recently demonstrated a facilitative role for lipid rafts in Akt pathway signaling and survival in LNCaP cells (31).

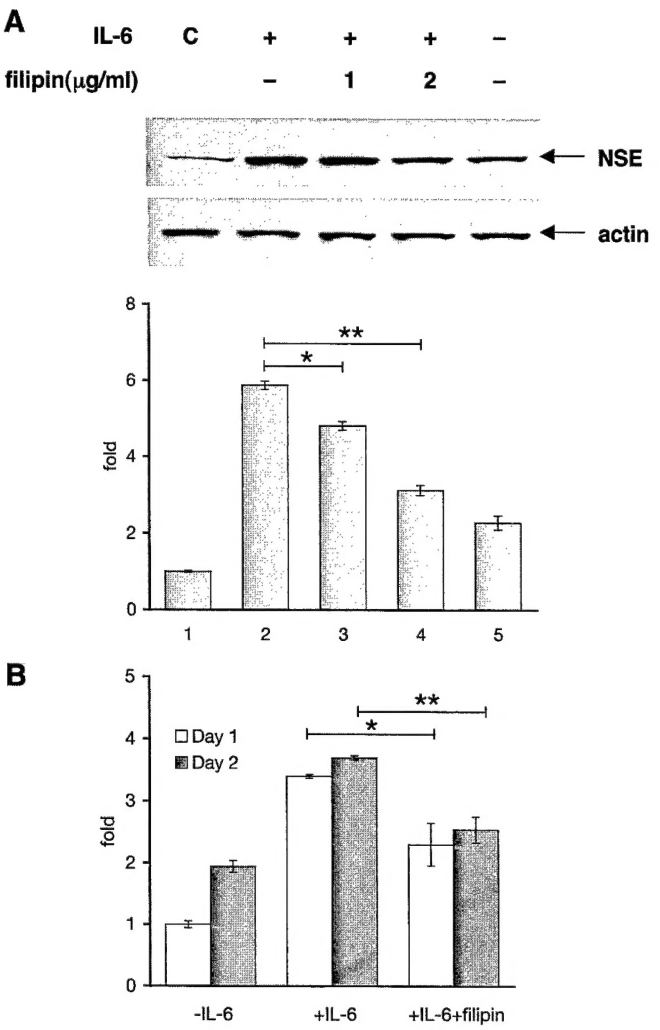

FIG. 5. NSE expression and NSE promoter activity are inhibited by disruption of plasma membrane rafts. Quiescent cells were preincubated with filipin for 1 h, then medium was changed to serum-free RPMI 1640 containing IL-6. A, To analyze the effect of filipin on NSE protein expression, total cell lysates were prepared 5d after IL-6 addition with or without the indicated quantity of filipin as shown, and Western blot was performed with anti-NSE and α-actin antibodies. Results shown in the graph are the means ± SD of NSE/actin ratios from three independent experiments (*, $P < 0.05$; **, $P < 0.005$). C (control), Serum-containing conditions (no IL-6). B, To determine the effect of filipin on NSE-promoter activity, LNCaP cells were transiently transfected with an NSE promoter-luciferase construct. Luciferase activity was measured under indicated conditions (−IL-6, +IL-6, and +IL-6+filipin) and at different times (1 and 2 d after IL-6 stimulation). Results are the means ± SD (n = 6; *, $P < 0.005$; **, $P < 0.05$).

A correlation between PCa aggressiveness and levels of STAT3 activation has been reported by several groups (39, 40). STAT up-regulation has also been shown to enhance the growth of LNCaP xenografts in an androgen-independent manner (41), consistent with a role for this signaling protein in hormone-refractory disease. Conversely, enforced down-regulation of STAT3 was shown to trigger apoptosis in PCa cell lines (40). We were able to significantly attenuate IL-6-mediated phosphorylation and nuclear translocation of STAT3, as well as increases in NSE promoter activity and protein levels, by disrupting the membrane raft compartment with filipin, a highly specific cholesterol-binding drug (24, 33, 34). These findings suggest an important role for membrane cholesterol as a mediator of STAT3-derived signal transduction events. The link between tumor cell survival and tumor aggressiveness, STAT3, and cholesterol suggests the possibility that membrane cholesterol may be an important component of the PCa cell's repertoire of defenses against apoptotic stimuli.

The hypothesis that cholesterol is a mediator of tumor cell survival is consistent with observations made decades ago, that benign and malignant prostate tissues accumulate cholesterol and other fatty deposits (42). Cholesterol synthesis inhibitors have also been demonstrated to induce apoptosis in prostate, mammary, neuroblastoma, and other tumor cell lines, suggesting a role for cholesterol or other downstream products of the mevalonate (cholesterol synthesis) pathway in resistance to apoptotic triggers (43–45). Furthermore, although the mechanism is still presently unknown, PCa incidence and progression have been linked to high fat diets and/or the consumption of animal products (46, 47). It is interesting that this epidemiological relationship to dietary fat and animal products is a feature of only some malignancies, with PCa and breast cancer being among the most notable (46). Similarly, apoptotic sensitivity to cholesterol synthesis inhibition has been demonstrated to be dependent on cell type or tissue origin (43).

The membrane cholesterol content of somatic cells in primates is partly dependent on low-density lipoprotein levels in the circulation. Long-term cholesterol-lowering drug therapy has been associated with a significant reduction in the incidence of a number of solid tumors, including PCa (48). One potential explanation for this finding is that cell survival signaling mechanisms involving cholesterol-rich membrane domains are affected by pharmacologic intervention to lower circulating cholesterol levels. Although more study is needed to rigorously test this hypothesis, an important corollary is that pharmacologic interventions directed at altering tumor cell cholesterol levels may be a rational means of therapy or chemoprevention for prostate and other solid tumors in which relationships to either cholesterol-rich diets or elevated cholesterol have been established.

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